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(54) Title: A RECEPTOR TYROSINE KINASE TARGET PROTEIN cDNA CLONING METHOD AND hGRB PROTEINS

(57) Abstract

A novel expression cloning method for detection and identification of proteins capable of binding to tyrosine-phosphorylated domains of receptor tyrosine kinases is based on the use of a novel probe. This probe comprises an amino acid sequence derived from the tyrosine-phosphorylated portion of the receptor molecule, or a functional derivative thereof, and has at least one phosphorylated tyrosine residue, lacks the tyrosine kinase portion of the receptor, and is detectably labeled. Also disclosed are a method for preparing the probe, a method for mapping to a chromosome a gene encoding a protein capable of binding to tyrosine-phosphorylated domains of receptor tyrosine kinases, and a method for purifying such a protein with the probe. Two proteins discovered using the above cloning method, GRB-1 and GRB-2, DNA encoding these proteins, and methods for detecting these proteins are also disclosed.

A RECEPTOR TYROSINE KINASE TARGET PROTEIN cDNA CLONING

METHOD AND hGRB PROTEINS

5   BACKGROUND OF THE INVENTION

Field of the Invention

The invention in the field of molecular and cell
biology relates to a novel method, based on direct expression
cloning, for identifying cellular proteins capable of binding
10 to the carboxy-terminal domain of receptor tyrosine kinases
and serving as substrates for this enzyme in major signal
transduction pathways. The invention also relates to novel
proteins identified using this method.

Description of the Background Art

15   A variety of polypeptide growth factors and hormones
mediate their cellular effects by interacting with cell
surface receptors having tyrosine kinase enzymatic activity
(for review, see Williams, L.T. et al., Science 243:1564-1570
(1989); Ullrich, A. et al., Cell 61:203-212 (1990); Carpenter,
20 G. et al. J. Biol. Chem. 265: 7709-7712 (1990)). The
interaction of these ligands with their receptors induces a
series of events which include receptor dimerization and
stimulation of protein tyrosine kinase activity. For the
epidermal growth factor receptor (EGFR) as well as other
25 receptors with tyrosine kinase activity, such as the platelet-
derived growth factor receptor (PDGFR), kinase activation and
receptor autophosphorylation result in the physical
association of the receptor with several cytoplasmic
substrates (Ullrich et al., supra).

30   Two substrates for the EGFR kinase have now been
definitively identified in living cells: (a) the
phosphatidylinositol specific phospholipase C-gamma (PLC-
gamma) and (b) the GTPase activating protein (GAP), a protein
which may be in the effector loop of the ras protein
35 (Margolis, B. et al. Cell 57: 1101-1107 (1989b); Meisenhelder,
J. et al. Cell 57: 1109-1122 (1989); Molloy, C.J. et al.

- 2 -

Nature 342: 711-714 (1989); Wahl, M.I. et al. J. Biol. Chem. 265: 3944-3948 (1990); Ellis, C. et al. Nature 343: 377-381 (1990); Kaplan, D.R. et al. Cell 61 121-133 (1990)).

5   Similarly, activated PDGFR was shown to tyrosine phosphorylate, and to become associated with PLC-gamma, GAP, and cellular tyrosine kinases such as pp60^{src} (Gould, K.L. et al., Molec. Cell. Biol. 8:3345-3356 (1988); Meisenhelder, J. et al., Cell 57:1109-1122 (1989); Molloy, C.J. et al., Nature 342:711-714 (1989); Kaplan, D.R. et al., Cell 61:121-133
10   (1990); Kazlauskas, A. et al., Science 247:1578-1581 (1990); Krypta, R.M. et al., Cell 62:481-492 (1990); Margolis, B. et al., Science 248:607-610 (1990)). While the exact sites responsible for the association of EGFR with either PLC-gamma or GAP have not been completely clarified, recent work has
15   begun to identify regions on both the substrate and receptor which contribute to the association.

SH2 (src homology 2) domains appear to be the regions responsible for the association of several tyrosine kinase substrates with activated growth factor receptors. SH2
20   domains are conserved sequences of about 100 amino acids found in cytoplasmic non-receptor tyrosine kinases such as pp60src, PLC-gamma, GAP and v-crk (Mayer, B.J. et al., Nature 332:272-275 (1988); Pawson, T. Oncogene 3:491-495 (1988)). While having distinct catalytic domains, all these molecules share
25   conserved SH2 and SH3 (src homology 3) domains and the ability to associate with receptors with tyrosine kinase activity (Anderson, D. et al., Science 250:979-982 (1990)).

Tyrosine kinase activation and receptor autophosphorylation are prerequisites for the association
30   between growth factor receptors and SH2 domain-containing proteins (Margolis, B. et al., Mol. Cell. Biol. 10:435-441 (1990);
Kumjian et al., Proc. Natl. Acad. Sci. USA 86:8232-8239 (1989); Kazlauskas, A. et al., Science 247:1578-1581 (1990)).
35   In particular, the carboxy-terminal (C-terminal) fragment of the EGFR, which contains all the known autophosphorylation sites, binds specifically to the SH2 domains of GAP and PLC-gamma (see below). Hence, a major site of association exists

- 3 -

between the SH2 domain of these substrate proteins and the
tyrosine phosphorylated C-terminal tail of the EGFR.

With the recognition that binding to the activated
tyrosine kinase receptor is conserved among several substrate
5 proteins, efforts to identify additional substrates which
share these properties have been undertaken. Target proteins
which bind to activated receptors have been identified by
analysis of proteins that co-immunoprecipitate with growth
factor receptors, or that bind to receptors attached to
10 immobilized matrices (Morrison, D.K. et al., Cell 58:649-657
(1989); Kazlauskas, A. et al., EMBO J. 9:3279-3286 (1990)).
While the identity of some of these proteins is known, several
others detected utilizing these approaches have not been fully
characterized. Moreover, it is possible that rare target
15 molecules which interact with activated receptors have not
been detected due to the limited sensitivity of these
techniques; the actual stoichiometry of binding may be low,
and the detergent solution necessary to solubilize proteins
may disrupt binding.

20   Conventional approaches to isolate and clone these
proteins have been arduous, requiring the use of large
quantities of tissue or cells lines to purify sufficient
amounts of protein for microsequence analysis and subsequent
conventional cDNA cloning. Therefore, a need for new
25 approaches for the cloning and subsequent isolation and
identification of e these proteins is recognized in the art.

SUMMARY OF THE INVENTION

One of the most pressing needs in our effort to
understand and gain control over the regulation of cell growth
30 and oncogenesis is having the ability to identify target
proteins for tyrosine kinases.

The present inventors have developed a novel
expression/cloning system for the rapid cloning of target
proteins for tyrosine kinase-type cellular receptors. The
35 cloning method is based on the ability of a certain class of
substrates to bind specifically to the tyrosine-phosphorylated
carboxy-terminus (C-terminus) of the epidermal growth factor

- 4 -

receptor (EGFR) (see Example VI, below; Margolis, B. et al.,
EMBO J. 9:4375-4380 (1990)).

The approach conceived by the present inventors has
important advantages over conventional cloning methods,
5 including avoidance of the laborious and costly task of
purifying potential target proteins for microsequencing
analysis. Moreover, the approach of the present invention
provides a method for identifying rare target molecules whose
association with activation receptors could not otherwise be
10 detected using conventional techniques. Furthermore, this
method allows the identification of structurally or
functionally related proteins which, though only weakly
homologous at the DNA level, are similar in their property of
binding to activated receptors with tyrosine kinase activity.
15 The latter ability is important since conventional screening
methods used to identify related genes are typically based on
low stringency nucleic acid hybridization. Indeed such
hybridization-based screening would not have been successful
in cloning and identifying the new proteins of the present
20 invention, GRB-1 and GRB-2, because of their lack of
similarity at the DNA level.

The present inventors' approach has its roots in
methods previously utilized to clone DNA-binding and jun-
binding proteins (Singh, H. et al., Cell 52:415-423 (1988);
25 MacGregor, P.F. et al., Oncogene 5:451-458 (1990)). The
methods of the present invention take advantage of the
inventors' conception and observation that the C-terminus of
the EGFR protein in which the tyrosine residues are
phosphorylated can bind substrates (see below). By creating a
30 labelled probe, wherein the tyrosines are phosphorylated with
^{32}P, the present inventors screened lambda gt11 cDNA
expression libraries from various human tissues and
identified numerous proteins which become associated with the
phosphorylated C-terminus of the EGFR. The present inventors
35 have termed the proteins discovered in this manner, "GRB" (for
Growth factor Receptor Bound). The cloning methodology of the
present invention has been designated, "CORT" (for Cloning Of
Receptor Targets).

- 5 -

The method of the present invention is proposed as a novel approach having both generality and rapidity for the identification and cloning of target molecules for tyrosine kinases.

5   The present invention is thus directed to a method for detecting expression of a protein, which is a target of a receptor tyrosine kinase, by a cell harboring an expression vector, the protein being capable of binding to a tyrosine-phosphorylated polypeptide portion of the receptor tyrosine

10   kinase, the method comprising:

(a)   contacting the cell, an extract thereof, a lysate thereof, or a supernatant thereof with a solid phase carrier, causing the binding of the protein to the carrier;

15   (b)   incubating the carrier-bound protein with the tyrosine-phosphorylated polypeptide, allowing the polypeptide to bind to the carrier-bound protein;

(c)   removing materials not bound to the carrier;

20   and

(d)   detecting the presence or measuring the amount of the tyrosine-phosphorylated polypeptide bound to the carrier,

thereby detecting the expression of the protein.

25   In a preferred embodiment, the receptor is the epidermal growth factor receptor, the platelet-derived growth factor receptor, or the fibroblast growth factor receptor.

This method is preferably performed using a prokaryotic cell, most preferably a bacterial cell such as

30   E. coli. The cell may also be eukaryotic, such as a yeast or a mammalian cell.

Preferably, the phosphorylated polypeptide is detectably labeled, such as with the radiolabel, ^{32}P.

The solid phase carrier is preferably a

35   nitrocellulose membrane, to which are transferred proteins released from lysed bacterial cells when a lambda gt11 library is being screened.

- 6 -

The present invention also provides a method for
mapping to a human chromosome a gene encoding a protein which
is capable of binding to a tyrosine-phosphorylated polypeptide
portion of a receptor tyrosine kinase molecule, the method
5 comprising:

  (a) infecting bacterial cells with a human gene
    expression library;

  (b) detecting a clone expressing the protein using
    a method according to claim 1;

10   (c) sequencing the DNA of the clone; and

  (d) mapping the sequence to a human chromosome.

The present invention is also directed to a
polypeptide probe useful in the detection of the expression of
a protein capable of binding to a tyrosine-phosphorylated

15 polypeptide portion of a receptor tyrosine kinase. The probe
comprises an amino acid sequence derived from the tyrosine-
phosphorylated portion of the receptor molecule,
or a functional derivative thereof, lacks the tyrosine kinase
domain, and the sequence must contain at least one

20 phosphotyrosine residue, preferably 4 or 5 phosphotyrosines.
The probe should be detectably labeled, preferably with ^{32}P.

  A preferred probe has between about 25 and 250 amino
acid residues.

  The probe of the present invention is useful for

25 detecting target proteins for receptor tyrosine kinases
including epidermal growth factor receptor, platelet-derived
growth factor receptor, and fibroblast growth factor receptor.

  The present invention also includes a method for
preparing the above probe, comprising

30   (a) providing the receptor, or a genetically
    engineered receptor-like derivative in
    substantially pure form, wherein the receptor
    or the receptor-like derivative has both a
    tyrosine kinase domain and a tyrosine-

35   phosphorylated domain, the tyrosine-
    phosphorylated domain including at least one
    tyrosine residue capable of being
    phosphorylated by the tyrosine kinase;

- 7 -

(b)   incubating the receptor or receptor-like
derivative with [gamma-^{32}P]adenosine
triphosphate under conditions permitting
phosphorylation of the tyrosine residue,

5   causing phosphorylation of the tyrosine residue
thereby producing the probe. In a preferred embodiment, the
method includes the step of:

(c)   additionally treating the phosphorylated
receptor molecule with an agent capable of

10   cleaving the molecule between the tyrosine
kinase domain and the tyrosine-phosphorylated
domain.

A preferred cleaving agent is cyanogen bromide.

In another embodiment, the above method involves a

15 genetically engineered receptor-like derivative which is a
polypeptide encoded by a DNA molecule comprising a DNA
sequence encoding tyrosine kinase, linked to a DNA sequence
encoding a selective enzymatic cleavage site, linked to a DNA
sequence encoding the tyrosine-phosphorylated domain, and

20 wherein the agent is an enzyme capable of cleaving at this
cleavage site. Preferred enzymes are Factor Xa and thrombin.

Also provided is a method for purifying from a
complex mixture a protein which is capable of binding to a
tyrosine-phosphorylated polypeptide portion of a receptor

25 tyrosine kinase molecule, the method comprising:

(a)   contacting the complex mixture with a solid
phase carrier to which a probe is bound,
allowing the protein to bind to the probe;

(b)   removing materials not bound to the carrier;

30   and

(c)   eluting the bound protein from the carrier,
thereby purifying the protein.

The present invention is also directed to a protein,
GRB-1, having the amino acid sequence shown in Figure 4. The

35 invention includes a protein, GRB-2, which includes the amino
acid sequence shown in Figure 17.

The invention is also directed to a DNA molecule
encoding the GRB-1 protein, and a DNA molecule encoding the

- 8 -

GRB-2 protein.   Included are DNA molecules encoding
functional derivatives of these proteins.   When the DNA
molecule naturally occurs, it is substantially free of the
nucleotide sequences with which it is natively associated.
5 The DNA molecules of this invention may be expression
vehicles, such as plasmids.

        Also provided is a host transformed with each of the
above DNA molecules.

        The present invention also includes a process for
10 preparing the GRB-1 or the GRB-2 protein substantially free of
other proteins with which each is natively associated,   or a
functional derivative thereof, comprising:

      (a)   culturing a host cell capable of expressing the
           protein under culturing conditions,

15   (b)   expressing the protein or functional
           derivative; and

      (c)   recovering the protein or functional derivative
           from the culture.

BRIEF DESCRIPTION OF THE DRAWINGS

20   Figure 1 is a filter blot pattern showing that the
carboxy-terminus of the EGFR interacts with GAP-SH2
immobilized on nitrocellulose filters. Bacterially-expressed
trpE/GAP-SH2 fusion protein or trpE as a control was spotted
at various concentrations onto nitrocellulose filters.   The
25 filters were hybridized overnight with $[^{32}P]$-labelled C-
terminal domain of the EGFR.   Autoradiography was for 2 hours.

        Figure 2 is a schematic diagram depicting the method
of cloning of receptor targets (CORT).   C-terminal domain of
the EGFR is phosphorylated with radiolabelled phosphorous.
30 Lambda gt11 library was plated at a density of 4×10^4 plaques
per 150 ml plate.   The plaques were overlaid with IPTG-
impregnated nitrocellulose filters for 12 hours, after which
the plaques were transferred to nitrocellulose and incubated
with the labelled probe.   Positive colonies are then selected
35 for further analysis.

        Figure 3 shows autoradiograms of phage expressing
GRB-1 protein.   A) Primary screen demonstrating one positive

- 9 -

signal (arrow) out of 40,000 phage plated.   B) Plaque
purification of phage expressing GRB-1.   All plaques bound to
the [^{32}P]-labelled C-terminal domain of the EGFR.

       Figure 4 shows the DNA sequence and predicted amino
5 acid sequence of GRB-1.   The protein has 724 amino acid
residues.

       Figure 5 compares the sequences of the SH2 domains
of GRB-1 with other proteins with similar motifs.   A) SH2
domains of GRB-1, c-src, v-abl, bovine PLC-gamma, GAP, and V-
10 crk.   N and C refer to N-Terminal and C-terminal SH2 domains
respectively.   Conservation amino acid substitutions are as
defined by Schwartz and Dayhoff: (A,G,P,S,T); (L,I,V,M);
(D,E,N,Q); (K,R,H); (F,Y,W); and C.   Bold letters identify
those position were the same or a conservative amino acid
15 substitution is present at 5 or more position.   Boxes identify
conserved motifs. B) A similar comparison of the SH3 domain of
GRB-1.

       Figure 6 is a schematic diagram comparing the
structural organization of the SH2 and SH3 domains. The scheme
20 includes known proteins containing SH2 and SH3 domains, such
as c-src, v-crk, PLC-gamma, GAP1 and GRB-1.

       Figure 7 is a Northern blot of monkey mRNA with GRB-
1 probe.   5ug of poly (A)+ mRNA, obtained from various monkey
tissue, was electrophoresed on 1.2%/2.2M agarose-formaldehyde
25 gel.   The blot was hybridized with a [32P]-nick translated DNA
probe corresponding to the insert from clone ki4.

       Figure 8 is a gel pattern showing that antibodies to
GRB-1 immunoprecipitate a protein of 85 kDa from
biosynthetically labelled cells.   Cells were metabolically
30 labelled with [^{35}S]methionine, after which lysates were
prepared and immunoprecipitated with either immune (I) or
preimmune (P) serum.   The immunoprecipitated protein was
separated on a 8% SDS/PAGE.   Autoradiography was performed
overnight.   Cell lines used include human glioblastoma cell
35 line, U1242, rat bladder carcinoma cell line, NBT-II and NIH-
3T3 cells.

       Figure 9 depicts several wild-type and mutant
proteins used in the studies. (A) EGF receptor constructs with

- 10 -

their known or predicted autophosphorylation sites. Wild-type
(W.T.), Kinase negative (K721A), and carboxy-terminal deletion
(CD126), were immunoprecipitated from previously described
transfected NIH373 cells expressing -300,000 EGF receptors.
5 EGFR-C represents a deletion mutant containing the cytoplasmic
domain of the EGF receptor produced by baculovirus-infected
SF9 cells. (B) Structure of PLC-gamma and trpE/GAP SH2
proteins indicating location of the SH2 and SH3 domains and
PLC-gamma tyrosine phosphorylation sites.

10   Figure 10 is a gel pattern showing association of
PLC-gamma with EGFR mutants. Wild-type (HER14), carboxy-
terminal deletion (DC126), or kinase-negative (K721A) EGFR
were immunoprecipitated with anti-EGFR mAb108. Receptors were
autophosphorylated with [gamma-^{32}P-ATP. Concomitantly EGFR-C
15 was added to protein A-Sepharose beads alone or to
immunoprecipitated K721A receptors either with or without ATP.
After further washes to remove ATP, lysate from - 15 x 10 6
PLC-gamma overexpressing 3T-P1 cells was added and mixed for
90 min at 4 C. After washing to remove unbound PLC-gamma,
20 proteins were separated on a 6% SDS-gel and transferred to
nitrocellulose for immunoblotting. One eighth of the sample
was utilized for anti-PTyr blotting, the remainder for anti-
PLC-gamma blotting (exposure time 14 h).
        Figure 11 is a gel pattern showing that
25 phosphorylation of PLC-gamma reduces its binding to the EGF
receptor. Full length EGFR was immunoprecipitated with
mAb108, and allowed to autophosphorylate. Lysate from PLC-
gamma overexpressing 3T-P1 cells was added and mixed for 90
min at 4°C. After binding, ATP was added to one half of the
30 samples allowing the PLC-gamma molecules to be phosphorylated
by the EGF receptor. SDS-PAGE sample buffer was then added to
one half of the EGFR-PLC-gamma complexes (NO WASH, left panel)
and directly loaded onto the 6% gel. The other half was
washed three times with HNTG and then loaded on the gel (WASH,
35 right panel. After running duplicate samples on SDS-PAGE, the
proteins were transferred to nitrocellulose and probed with
anti-PLC-gamma and [^{125}I]protein A. The bands were
subsequently cut from the nitrocellulose and quantitated in a

gamma counter. After three washes with HNTG, 50±5% (Mean±SEM,
n = 4) of the non-phosphorylated PLC-gamma remained bound to
the EGFR while only 22±4% of the phosphorylated PLC-gamma
remained (exposure time: 12h).

5   Figure 12 is a gel pattern showing binding of EGFR-C
to trpE proteins. (A) EGFR-C (0.5 μg) was immunoprecipitated
with antibody C and washed. MnCl$_2$ alone or MnCl$_2$ and ATP were
then added to facilitate autophosphorylation. trpE or
trpE/GAP SH2 (approximately 2 μg). The immunoprecipitates
10 were separated on a 10% SDS-gel, transferred to nitrocellulose
and immunoblotting was performed with anti-trpE. For
comparison, about 0.1 μg of trpE or trpE/GAP SH2 lysate was
loaded directly on to the gel (right panel of A). (B) trpE or
trpE/GAP SH2 was immunoprecipitated with anti-trpE antibodies
15 and washed. Phosphorylated or non-phosphorylated EGFR-C (0.5
μg) was then added and allowed to bind as above. After
washing, samples were separated on a 10% gel, transferred to
nitrocellulose and probed with antibody C. The two samples on
the right represent 0.5 μg of phosphorylated and non-
20 phosphorylated kinase loaded directly onto the gel (exposure
time: 2 h).

            Figure 13 is a gel pattern showing binding of
trpE/GAP SH2 to wild-type and mutant EGFR. (A) Wild-type
receptor (HER14) or the carboxy-terminal deletion CD126
25 receptor were immunoprecipitated with mAb 108. MnCl$_2$ alone or
MnCl$_2$ and ATP were then added to the autophosphorylated half
of the receptor-containing samples. One set of CD126 was also
cross-phosphorylated with 0.5 μg of EGFR-C. trpE/GAP SH2 was
then added for 90 min at 4°C and, after three more washes,
30 loaded onto SDS-PAGE. After transfer to nitrocellulose, blots
were probed with anti-trpE (left panel), anti-EGFR RK2 (center
panel), or anti-PTyr (right panel). RK2 and anti-PTyr are
both 1/8 of the total sample and were separated on 7% SDS-
PAGE. The remaining sample was loaded on a 10% gel for the
35 anti-trpE blot (exposure time 14 h). (B) Lysates from NIH3T3
2.2 cells containing no EGFR (3T3) or from cells with kinase-
negative receptors (K21A) were immunoprecipitated with mAb108.
To all immunoprecipitates, 0.5 μg of EGFR-C was added and then

- 12 -

MnCl$_2$ alone or MnCl$_2$ and ATP. trpE/GAP SH2 was added and samples prepared and immunoblotted as in (A) (exposure time: 19 h).

Figure 14 is a gel pattern showing binding of PLC-
5 gamma and trpE/GAP SH2 to the CNBr cleaved C-terminal fragment of EGFR. EGFR-C (10 μg) was incubated in a Centricon 30 in 20 mM HEPES, pH 7.5 with 100 μg BSA as a carrier protein. The phosphorylated and non-phosphorylated EGFR-C were then each divided in two with one half being stored in buffer while the
10 other half was cleaved with CNBr. The four samples either with or without ATP, and with or without CNBr were then each brought up in 500 ul 1% Triton X-100 lysis buffer, split in two, and immunoprecipitated with anti-C antibody. After washing the immunoprecipitates, lysates containing PLC-gamma
15 or trpE/GAP SH2 were added. Immunoblotting was then performed on the samples as above with anti-trpE or anti-PLC-gamma. For the right panel, a fraction of the cleaved and uncleaved EGFR-C (0.1 μg) was loaded directly on the gel without immunoprecipitation and immunoblotted with RK2 (exposure time
20 14 h). The dark band seen in all lines of the anti-trpE blot runs at about 40 kDa (also seen in Figure 13) and represents [^{125}I]protein A binding to the heavy chain of the immunoprecipitating antibody.

Figure 15 is a gel pattern showing binding of the
25 tyrosine phosphorylated C-terminal EGFR fragment to trpE/GAP SH2 but not to trpE. EGFR-C (5 μg) was autophosphorylated by the addition of [gamma-32P]ATPl. The phosphorylated EGFR-C was concentrated in a Centricon 30, and then cleaved with CNBr in 70% formic acid. One half of the sample (350,000 c.p.m.)
30 was allowed to bind to trpE or trpE/GAP SH2 as in Figure 12B, washed and run on a 10% SDS-gel. (A) Binding of phosphorylated CNBr cleaved EGFR-C to trpE (B) Binding of phosphorylated CNBr cleaved EGFR-C to trpE GAP SH2 (C) 3000 c.p.m. of CNBr- cleaved EGFR-C (D) for comparison 3000 c.p.m.
35 of cleaved EGFR-C (exposure time 20 h). EGFR 984/1186 indicates the sequence of the tyrosine autophosphorylated fragment generated by CNBr.

- 13 -

Figure 16 shows the partial nucleotide sequence and predicted amino acid sequence of GRB-2.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors have developed a novel probe
5 and method using this probe for rapid expression cloning of DNA encoding proteins which have the characteristic of binding to the tyrosine-phosphorylated C-terminal tail a receptor tyrosine kinase molecule, in particular the epidermal growth factor receptor (EGFR).

10   By the term "expression" is intended the transcription and translation of a DNA sequence encoding a protein, to yield a protein molecule. Expression cloning is a method wherein the DNA being cloned encodes a protein. The desired DNA, typically in the form of a cDNA library, is
15 detected by means of its expression and direct detection of the protein which it encodes. Expression cloning systems are well-known in the art (see: Sambrook, J. et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), which is hereby
20 incorporated by reference). Typically, the protein is expressed from a library such as a lambda gt11 library, via infection of bacteria with the lambda gt11 expression vector. The expressed protein, released into the area of the bacteriophage plaque, is transferred to a nitrocellulose
25 filter and typically detected using an antibody to stain the filter. For novel proteins, or proteins which are not available in sufficient quantities to prepare an antibody, or proteins which do not have a known ligand for affinity-based detection, such conventional expression cloning methods cannot
30 be used.

The expression cloning method of the present invention may be easily applied to many other receptor systems. For example, certain target molecules bind to the tyrosine phosphorylated portion of PDGFR and the colony
35 stimulating factor-1 (CSF-1) (Coughlin, S.R. et al., Science 243:1191-1194 (1989); Kazlauskas, A. et al., Cell 58:1121-1133 (1989); Shurtleff, S.A. et al., EMBO J. 9:2415-2421 (1990);

- 14 -

Reedjik, M. et al., Mol. Cell. Biol. 10:5601-5608 (1990)). In
these two receptors, the tyrosine phosphorylation occurs in a
kinase insert domain, rather than in the C-terminal domain as
is the case with the EGFR. Therefore, specific probes

5 utilizing the kinase insert domain, or a functional derivative
thereof (defined below), of either the PDGFR or CSF-1 receptor
can be similarly used for expression cloning. Similar probes
can also be constructed for the fibroblast growth factor (FGF)
receptor (which is tyrosine phosphorylated in the C-terminal

10 domain) or the HER 2/neu receptor, both of the which are also
able to interact with SH2 containing proteins such as PLC-
gamma. In other receptors, such as the insulin receptor,
tyrosine phosphorylation occurs in the kinase domain itself.

Thus, while it will be appreciated that different

15 sites are tyrosine-phosphorylated in different proteins, e.g.,
the C-terminal domain in the EGFR, the kinase domain in
insulin receptor, and a kinase domain insert in PDGFR, the
present invention recognizes the common features of all these
structures, the presence of one or more phosphotyrosine

20 residues, and the ability of certain cellular proteins to bind
on the basis of affinity to a polypeptide containing one or
more phosphotyrosines. While reference will generally be made
below to a probe which is a C-terminal domain, with reference
to the EGFR, this language is not intended to be limiting and

25 is intended to include all of the other alternative tyrosine-
phosphorylated domains discussed above.

The methods and approach of the present invention
can be applied to the cloning and identification of all target
molecules which are capable of interacting in a specific

30 manner with tyrosine phosphorylated polypeptides, such as the
activated phosphorylated receptors described above.
Additional proteins which bind to tyrosine-phosphorylated
sequences, such as the tyrosine-specific phosphatases, e.g. R-
PTPases (Sap, J. et al., Proc. Natl. Acad. Sci. USA 87:6112-

35 6116 (1990); Kaplan, R. et al., Proc. Natl. Acad. Sci. USA
87:7000-7004 (1990)). The methods are also applicable in the
cloning and identification of proteins which bind to

- 15 -

phosphorylated serine/threonine residues, as with
serine/threonine-specific phosphatases.

Use of the probe of the present invention allows the
rapid cloning of DNA and identification of the encoded
5 proteins from a gene expression library. The method is
particularly useful with a bacteriophage lambda gt11 library.
Screening a human fetal brain lambda gt11 expression library
has permitted the present inventors to clone two genes and to
characterize the proteins they encode. One, termed GRB-1, was
10 fully sequenced and found to encode novel human protein with a
molecular weight of about 85 kDa which contained two SH2
domains and one SH3 domain (Figure 4 and Figure 5). GRB-2,
which was partially sequenced, also contains unique SH2 and
SH3 domains.

15 SH2 domains, such as in the GAP and PLC-gamma
proteins, are responsible for the association of these
proteins with the phosphorylated C-terminus of the EGFR (see
Example VI, below). Thus, one function of SH2 domains is to
juxtapose the intracellular portion of receptor tyrosine
20 kinase molecules with their substrates to facilitate efficient
tyrosine phosphorylation.

Detailed analysis of one of the cDNA clones of the
present invention, GRB-1, identified using the methods of the
invention, reveals a novel sequence containing two SH2 domains
25 and one SH3 domain. This protein is expressed in various
tissues and cell lines. Its predicted molecular weight, 85
kDa, is consistent with its migration upon sodium dodecyl
sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

By the term "receptor tyrosine kinase" is intended a
30 transmembrane protein having an extracellular receptor domain,
and one or more intracellular domains, including a domain
having tyrosine kinase enzymatic activity. Additional
intracellular domains may have sequence homology to SH2.
These molecules are well known in the art (Williams, L.T. et
35 al., Science 243:1564-1570 (1989); Ullrich, A. et al., Cell
61:203-212 (1990); Carpenter, G. et al. J. Biol. Chem. 265:
7709-7712 (1990)).

- 16 -

The proteins which interact with, and which may be phosphorylated by, the receptor tyrosine kinases are referred to as "target" proteins for these receptors, in distinction from the "ligands" for these receptors, which bind to the

5   extracellular receptor domain.

According to the present invention, the expression cloning method is performed directly on a gene expression library, such as lambda gt11. In a preferred embodiment, the DNA is human cDNA. More preferably, the DNA is human fetal

10 brain DNA. Using such a source as the starting material for the cloning of human genes has a great advantage over the alternative means, in which a large amount of tissue is taken, and antibodies produced, or the protein purified and partially sequenced, and oligonucleotide probes prepared from this

15 sequence and used to screen a genomic DNA or cDNA library. The advantage of bypassing these steps is of most relevance in the case of human genes, since tissue is generally not available in large quantities, with the exception of placenta.

The expression library is screened in a single step.

20 Preferably, the lambda plaques are blotted onto a solid carrier, preferably nitrocellulose, allowing the transfer of library DNA-encoded proteins which are expressed in the infected bacteria and transferred to the carrier. This carrier is then incubated with the probe of the present

25 invention, as described herein. The probe is allowed to bind to proteins which have the capability of binding to the tyrosine-phosphorylated polypeptide. Based on the label used in the probe, preferably the radioisotope ^{32}P, an appropriate detection system is used to identify the plaques containing

30 the protein of interest. The phage in these plaques are then selected, and the DNA inserts in them can be re-cloned, excised and placed into other vectors, used for large scale expression of the protein, and the like, as is well-known in the art.

35 One of ordinary skill in the art will appreciate that the concentrations, times, temperatures can be varied depending on the precise nature of the system used, and will know how to vary the appropriate parameters without undue

- 17 -

experimentation. Furthermore, general methods in this area
are set forth in Sambrook et al. (supra).

Materials of which solid phase carrier can be made
include, but are not limited to, nitrocellulose, cellulose,
5 paper, substituted polystyrenes, acrylonitriles,
polycarbonate, polypentene, or silicone oxide.

The probe of the present invention is a tyrosine-
phosphorylated polypeptide molecule derived from the C-
terminal domain of a receptor tyrosine kinase, preferably the
10 EGFR. The polypeptide can have between about 25 and about 250
amino acids in length. The probe can be a phosphorylated
native sequence or a functional derivative thereof (defined
below). Highly efficient phosphorylation is obtained by
using the tyrosine kinase domain present on the receptor
15 molecule to autophosphorylate the C-terminal region at between
1 and 5 tyrosine residues. Known methods and conditions
(described in detail in Example I) are used to phosphorylate
the tyrosine residues. A preferred substrate is [gamma-P^{32}-
adenosine triphosphate). The source of the receptor molecule
20 used as the source material to make the probe can include
molecules chemically purified from tissues or cells, or
molecules produced recombinant DNA methods.

When using recombinant techniques, a native receptor
may be produced, or alternatively, a receptor-like derivative
25 may be produced. A preferred receptor-like derivative
includes the tyrosine kinase domain linked to the C-terminal
domain. In another embodiment, the two domains may be
produced as separate molecules, and mixed together to achieve
tyrosine phosphorylation of the C-terminus-derived
30 polypeptide.

The probe comprising a tyrosine-phosphorylated C-
terminal portion of the receptor tyrosine kinase, as described
herein can be produced by recombinant means in the form of a
fusion protein.

35   As used herein, a "fusion protein" may refer to a
fused protein comprising a bacterial protein and a polypeptide
of interest such as a protein having an SH2 domain.
Alternatively, a fusion protein may also be an artificially

- 18 -

constructed receptor tyrosine kinase-like derivative, wherein
a DNA sequence encoding the tyrosine kinase domain has been
linked to a selective enzymatic cleavage site, which, in turn,
is linked to a receptor tyrosine kinase C-terminal domain
5 having one or more tyrosine residues which can be
phosphorylated by the kinase. Such a genetic construct
encoding this type of "fusion protein" can be inserted into an
expression vehicle and expressed in a bacterial or eukaryotic
host. Once expressed, such a fusion protein can be allowed to
10 autophosphorylate, wherein the kinase acts to phosphorylate
the tyrosine residues in the C-terminal domain. Following
this phosphorylation, use of the appropriate enzyme will
cleave at the selective cleavage site, thus separating the N-
terminal kinase from the C-terminal phosphorylated
15 polypeptide, which can now serve as a probe.

Itakura et al. (Science 198:1056-1063 (1977)) and
Riggs (U.S. Patent 4,366,246 (1982)) taught methods for
bacterial expression of a foreign protein, in the form of a
fusion protein, which prevents intracellular degradation of
20 the foreign protein. Furthermore, overexpressed fusion
proteins are frequently deposited as inclusion bodies in the
bacterial cells, and are therefore easier to isolate and
purify (Marston, Biochem. J. 240:1-12 (1986)). These
references suggested alternate cleavage methods based on the
25 concept of selective cleavage sites: Just as methionine could
be inserted at the desired junction to serve as a target site
for cyanogen bromide, so too could specific amino acid sites
be introduced which could be attacked by proteolytic enzymes
with amino acid specificity. Nagai et al. (Nature 309:810-812
30 (1984) disclosed the production of a desired gene product in a
fusion protein by introducing a sequence of 4 amino acids
which serve as a cleavage site for the blood coagulation
factor Xa. Cleavage by Factor Xa also eliminated the extra N-
terminal methionine encoded by the initiation codon which is
35 present in most eukaryotic proteins expressed in E. coli by
conventional methods. Other references describing the use of
enzymatic cleavage sites recognized by Factor Xa or thrombin,
for enhanced expression in bacteria and greater ease of

- 19 -

purification of the desired protein (Germino et al., Proc.
Natl. Acad. Sci. USA 81:692-4696 (1984); Scholtissek et al.,
Gene 62:55-64 (1988); Smith et al., Gene 67:31-40 (1988);
Knott et al., Eur. J. Biochem. 174:405-410 (1988); and Dykes
5 et al., Eur. J. Biochem. 174:411-416 (1988)).

The term "selective cleavage site" refers to an
amino acid residue or residues which can be selectively
cleaved with either chemicals or enzymes and where cleavage
can be achieved in a predictable manner. A selective
10 enzymatic cleavage site is an amino acid or a peptide sequence
which is recognized and hydrolyzed by a proteolytic enzyme.
Examples of such sites include trypsin or chymotrypsin
cleavage sites. In a preferred embodiment of this invention,
the selective cleavage site is comprised of the sequence Ile-
15 Glu-Gly-Arg, which is recognized and cleaved by blood
coagulation factor Xa. In another embodiment, the selective
cleavage site has the sequence Leu-Val-Pro-Arg, which is
recognized and cleaved by thrombin.

In constructing the receptor-like derivative, an
20 oligonucleotide sequence, 5' to the sequence coding for the
enzyme recognition site can be included, and may vary in
length. For example, in one embodiment, 13 nucleotides are
situated between the codon for Ile (the start of the factor Xa
recognition site) and the 3' end of the sequence encoding the
25 tyrosine kinase domain.

Thus, in one embodiment of the present invention,
the Ile-Glu-Gly-Arg sequence is introduced between the
tyrosine kinase domain and the C-terminal domain. In another
embodiment, the Leu-Val-Pro-Arg sequence is introduced. The
30 proteins having this cleavage site are expressed in bacteria
using standard methods. Thereafter, autophosphorylation of
the C-terminal domain, preferably with [gamma32P] adenosine
triphosphate, is allowed to occur, followed by selective
cleavage of the tyrosine-phosphorylated C-terminal domain with
35 the appropriate cleaving agent, e.g. factor Xa.

The present invention also provides a method for
mapping a gene, preferably a human gene, which encodes a
target protein for a receptor tyrosine kinase (such as a GRB

- 20 -

protein as defined herein), to a particular human chromosome.
This method combines the new expression cloning method
described herein with one of several known techniques for
mapping a gene to a particular chromosome. Thus, according to
5 the present invention, a clone, such as a lambda gt11 clone,
containing a DNA insert encoding a GRB protein, is identified
using the expression cloning methods disclosed herein. The
insert may be further subcloned, if desired, using methods
well-known in the art, and a probe constructed, either by
10 direct labeling of the nucleic acid of the clone or by
producing an oligonucleotide probe corresponding to a unique
portion of the clone's sequence (see: Sambrook, J. et al.
(Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold
Spring Harbor Press, Cold Spring Harbor, NY (1989)). This
15 labeled probe can is then used in a hybridization assay with
commercially available blots, such Chromosome Blots from Bios
Corporation (New Haven, Connecticut) which contain DNA from a
panel of human-hamster somatic cell hybrids (Kouri, R. E. et
al., Cytogenet. Cell Genet. 51:1025 (1989)). By comparison of
20 which human chromosomes remain in the human-hamster hybrid
cell and the hybridization of the probe specific for the GRB
gene of interest, the gene is mapped to a particular human
chromosome. In this way, linkage is established to known
human genes (or diseases caused by mutations therein) present
25 on this chromosome. Using methods well-known in the art for
finer mapping, e.g., using known human deletion mutations, the
GRB gene can be mapped more precisely to other human genes.
        The tyrosine-phosphorylated receptor tyrosine kinase
C-terminal probe polypeptide of the present invention, as well
30 as the GRB proteins of the present invention, and additional
yet unknown GRB proteins which are discovered using the
methods of this invention, are useful in methods for screening
drugs and other agents which are capable of modulating cell
growth control that occurs via signal transduction through
35 receptor tyrosine kinases. By attaching a tyrosine-
phosphorylated probe polypeptide or a GRB protein, or
fragments thereof, to a solid phase carrier matrix, an
affinity probe is created which can be used to isolate and

- 21 -

purify molecules from complex mixtures which are capable of
binding to the affinity probe. Furthermore, such an affinity
probe is useful for detecting the presence in a biological
fluid of a molecule capable of binding the tyrosine-
5 phosphorylated probe or the GRB protein. Similarly, chemical
agents can be tested for their capacity to interact with the
probe or GRB.

        Methods for coupling proteins and peptides to the
solid phase, the solid phase substances useful in these
10 methods, and means for elution, are well known to those of
skill in the art.

        In the case of growth factor receptors which are
receptor tyrosine kinases, including EDGFR, PDGFR and FGFR,
tyrosine phosphorylation is linked to cell growth and to
15 oncogenic transformation. Disruption of the action of a GRB
in the cell may prevent or inhibit growth, and might serve as
means to counteract development of a tumor. Furthermore, a
mutation in the C-terminal portion of the receptor tyrosine
kinase or the GRB, or a dysregulation in their mutual
20 interactions, may promote susceptibility to cancer

        The insulin receptor (InsR) is also a receptor
tyrosine kinase, and tyrosine phosphorylation in cells bearing
InsR is associated with normal physiological function. In
contrast to the case of cell growth and cancer, disruption of
25 normal interactions between of the tyrosine-phosphorylated
portion of the receptor and the GRB would counteract insulin
effects. Subnormal levels or activity of a GRB protein may
act to remove a normal counterregulatory mechanisms. Perhaps
overexpression or overactivity of a GRB protein could inhibit
30 or totally prevent the action of insulin on cells, leading to
diabetes (of an insulin-resistant variety). Thus
susceptibility to diabetes may be associated with GRB-1 or
GRB-2 protein dysregulation.

        Therefore methods of the present invention for
35 identifying normal or mutant GRB protein genes, or for
detecting the presence or the amount of GRB-1 or GRB-2 in a
cell can serve as methods for identifying susceptibility to
cancer, diabetes, or other diseases associated with

alterations in cellular metabolism mediated by receptor
tyrosine kinase pathways.

The present invention provides methods for
evaluating the presence, and the level of normal or mutant
5 GRB-1 or GRB-2 protein in a subject. Altered expression of
these proteins, or presence of a mutant GRB protein, in an
individual may serve as an important predictor of
susceptibility to oncogenic transformation and the development
of cancer. Alternatively, altered expression of GRB protein
10 may serve as an important predictor of susceptibility to
diabetes.

Oligonucleotide probes encoding various portions of
the GRB protein are used to test cells from a subject for the
presence DNA or RNA sequences encoding the GRB protein. A
15 preferred probe would be one directed to the nucleic acid
sequence encoding at least 4 amino acid residues, and
preferably at least 5 amino acid residues of the GRB-1 or GRB-
2 protein of the present invention. Qualitative or
quantitative assays can be performed using such probes. For
20 example, Northern analysis (see Example III, below) is used to
measure expression of an GRB protein mRNA in a cell or tissue
preparation.

Such methods can be used even with very small
amounts of DNA obtained from an individual, following use cf
25 selective amplification techniques. Recombinant DNA
methodologies capable of amplifying purified nucleic acid
fragments have long been recognized. Typically, such
methodologies involve the introduction of the nucleic acid
fragment into a DNA or RNA vector, the clonal amplification of
30 the vector, and the recovery of the amplified nucleic acid
fragment. Examples of such methodologies are provided by
Cohen et al. (U.S. Patent 4,237,224), Sambrook et al. (supra),
etc.

Recently, an in vitro, enzymatic method has been de-
35 scribed which is capable of increasing the concentration of
such desired nucleic acid molecules. This method has been
referred to as the "polymerase chain reaction or "PCR"
(Mullis, K. et al., Cold Spring Harbor Symp. Quant. Biol.

- 23 -

51:263-273 (1986); Erlich H. et al., EP 50,424; EP 84,796, EP
258,017, EP 237,362; Mullis, K., EP 201,184; Mullis K. et al.,
US 4,683,202; Erlich, H., US 4,582,788; and Saiki, R. et al.,
US 4,683,194).

5   The polymerase chain reaction provides a method for
selectively increasing the concentration of a particular
nucleic acid sequence even when that sequence has not been
previously purified and is present only in a single copy in a
particular sample. The method can be used to amplify either
10 single- or double-stranded DNA. The essence of the method
involves the use of two oligonucleotide probes to serve as
primers for the template-dependent, polymerase mediated
replication of a desired nucleic acid molecule.

          The precise nature of the two oligonucleotide probes
15 of the PCR method is critical to the success of the method.
As is well known, a molecule of DNA or RNA possesses
directionality, which is conferred through the 5'-3' linkage
of the phosphate groups of the molecule. Sequences of DNA or
RNA are linked together through the formation of a
20 phosphodiester bond between the terminal 5' phosphate group of
one sequence and the terminal 3' hydroxyl group of a second
sequence. Polymerase dependent amplification of a nucleic acid
molecule proceeds by the addition of a 5' nucleotide
triphosphate to the 3' hydroxyl end of a nucleic acid
25 molecule. Thus, the action of a polymerase extends the 3' end
of a nucleic acid molecule. These inherent properties are
exploited in the selection of the oligonucleotide probes of
the PCR. The oligonucleotide sequences of the probes of the
PCR method are selected such that they contain sequences
30 identical to, or complementary to, sequences which flank the
particular nucleic acid sequence whose amplification is
desired.

          More specifically, the oligonucleotide sequences of
the "first" probe is selected such that it is capable of
35 hybridizing to an oligonucleotide sequence located 3' to the
desired sequence, whereas the oligonucleotide sequence of the
"second" probe is selected such that it contains an
oligonucleotide sequence identical to one present 5' to the

desired region. Both probes possess 3' hydroxy groups, and
therefore can serve as primers for nucleic acid synthesis.
            In the PCR, the reaction conditions are cycled
between those conducive to hybridization and nucleic acid
5 polymerization, and those which result in the denaturation of
duplex molecules. In the first step of the reaction, the
nucleic acids of the sample are transiently heated, and then
cooled, in order to denature any double-stranded molecules
which may be present. The "first" and "second" probes are
10 then added to the sample at a concentration which greatly
exceeds that of the desired nucleic acid molecule. When the
sample is incubated under conditions conducive to
hybridization and polymerization, the "first" probe will
hybridize to the nucleic acid molecule of the sample at a
15 position 3' to the sequence to be amplified. If the nucleic
acid molecule of the sample was initially double-stranded,
the "second" probe will hybridize to the complementary strand
of the nucleic acid molecule at a position 3' to the sequence
which is the complement of the sequence whose amplification is
20 desired. Upon addition of a polymerase, the 3' ends of the
"first" and (if the nucleic acid molecule was double-stranded)
"second" probes will be extended. The extension of the
"first" probe will result in the synthesis of an
oligonucleotide having the exact sequence of the desired
25 nucleic acid. Extension of the "second" probe will result in
the synthesis of an oligonucleotide having the exact sequence
of the complement of the desired nucleic acid.
            The PCR reaction is capable of exponential
amplification of specific nucleic acid sequences because the
30 extension product of the "first" probe, of necessity, contains
a sequence which is complementary to a sequence of the
"second" probe, and thus can serve as a template for the
production of an extension product of the "second" probe.
Similarly, the extension product of the "second" probe, of
35 necessity, contains a sequence which is complementary to a
sequence of the "first" probe, and thus can serve as a
template for the production of an extension product of the
"first" probe. Thus, by permitting cycles of polymerization,

- 25 -

and denaturation, a geometric increase in the concentration of the desired nucleic acid molecule can be achieved. Reviews of the PCR are provided by Mullis, K.B. (Cold Spring Harbor Symp. Quant. Biol. 51:263-273 (1986)); Saiki, R.K., et al.
5 (Bio/Technology 3:1008-1012 (1985)); and Mullis, K.B., et al. (Meth. Enzymol. 155:335-350 (1987)).

        In one embodiment, the invention is directed to a naturally occurring GRB-1 and GRB-2 proteins. In another embodiment, the invention is directed to recombinant GRB-1 and
10 GRB-2 proteins. The invention provides the naturally occurring protein molecule substantially free of other proteins with which it is natively associated. "Substantially free of other proteins or glycoproteins" indicates that the protein has been purified away from at least 90 per cent (on a
15 weight basis), and from even at least 99 per cent if desired, of other proteins and glycoproteins with which it is natively associated, and is therefore substantially free of them. That can be achieved by subjecting the cells, tissue or fluids containing the GRB-1 or GRB-2 protein to standard protein
20 purification techniques such as immunoadsorbent columns bearing monoclonal antibodies reactive against the protein.

        The nucleotide sequence of the GRB-1 gene, and the amino acid sequence of the GRB-1 protein, are shown in Figure 4. The partial nucleotide sequence of GRB-2 and the partial
25 amino acid sequence, are shown in Figure 16.

        In a preferred embodiment, GRB-1 or GRB-2, or an unknown GRB protein, can be isolated and purified using as an affinity probe, the probe of the present invention which is a tyrosine-phosphorylated C-terminal domain of a receptor
30 tyrosine kinase, or a functional derivative thereof.

        Alternatively, the purification can be achieved by a combination of standard methods, such as ammonium sulfate precipitation, molecular sieve chromatography, and ion exchange chromatography.

35   It will be understood that the GRB-1 and GRB-2 proteins of the present invention can be biochemically purified from a variety of cell or tissue sources. For

- 26 -

preparation of naturally occurring GRB protein, tissues such
as mammalian placenta or brain are preferred.

      Alternatively, because the gene for GRB1 and GRB-2
can be isolated or synthesized, the polypeptide can be

5 synthesized substantially free of other proteins or
glycoproteins of mammalian origin in a prokaryotic organism or
in a non-mammalian eukaryotic organism, if desired. As
intended by the present invention, a recombinant GRB-1 or GRB-
2 molecule produced in mammalian cells, such as transfected

10 COS, NIH-3T3, or CHO cells, for example, is either a naturally
occurring protein sequence or a functional derivative thereof.
Where a naturally occurring protein or glycoprotein is
produced by recombinant means, it is provided substantially
free of the other proteins and glycoproteins with which it is

15 natively associated.

      Alternatively, methods are well known for the
synthesis of polypeptides of desired sequence on solid phase
supports and their subsequent separation from the support. In
particular, the tyrosine-phosphorylated C-terminal domain

20 probe of the present invention, or a functional derivative
thereof, can be synthesized using a peptide synthesis method
wherein phosphotyrosine is provided in place of tyrosine,
resulting in direct synthesis of the phosphorylated form of
the polypeptide.

25   The present invention provides "functional
derivatives" of the tyrosine-phosphorylated C-terminal domain
polypeptide and or the GRB-1 and GRB-2 proteins.

      By "functional derivative" is meant a "fragment,"
"variant," "analog," or "chemical derivative" of the GRB

30 protein, which terms are defined below. A functional
derivative retains at least a portion of the function of the
native protein which permits its utility in accordance with
the present invention.

      A "fragment" of any of the proteins or polypeptides

35 of the present invention refers to any subset of the molecule,
that is, a shorter peptide.

      A "variant" of the protein refers to a molecule sub-
stantially similar to either the entire peptide or a fragment

thereof. Variant peptides may be conveniently prepared by direct chemical synthesis of the variant peptide, using methods well- known in the art.

Alternatively, amino acid sequence variants of the
5 peptide can be prepared by mutations in the DNA which encodes the synthesized peptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence. Any combination of deletion, insertion, and substitution may also be made to arrive at the
10 final construct, provided that the final construct possesses the desired activity. Obviously, the mutations that will be made in the DNA encoding the variant peptide must not alter the reading frame and preferably will not create complementary regions that could produce secondary mRNA structure (see
15 European Patent Publication No. EP 75,444).

At the genetic level, these variants ordinarily are prepared by site-directed mutagenesis (as exemplified by Adelman et al., DNA 2:183 (1983)) of nucleotides in the DNA encoding the peptide molecule, thereby producing DNA encoding
20 the variant, and thereafter expressing the DNA in recombinant cell culture (see below). The variants typically exhibit the same qualitative biological activity as the nonvariant peptide.

An "analog" of the tyrosine-phosphorylated
25 polypeptide or the GRB protein refers to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

A "chemical derivative" of the tyrosine-phosphorylated polypeptide or the GRB protein contains addi-
30 tional chemical moieties not normally a part of the peptide. Covalent modifications of the peptide are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable
35 of reacting with selected side chains or terminal residues.

Cysteinyl residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or

- 28 -

carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, alpha-bromo- beta-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N- alkylmaleimides, 3-nitro-2-pyridyl disulfide,

5 methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4- nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is

10 relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization

15 with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride;

20 trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal,

25 2,3- butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine

30 epsilon-amino group.

The specific modification of tyrosyl residues per se has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most

35 commonly, N-acetylimidizol and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

- 29 -

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1- ethyl-3-(4-azonia-4,4-dimethylpentyl)

5   carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl

10 residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Derivatization with bifunctional agents is useful for cross-linking the peptide to a water-insoluble support

15 matrix or to other macromolecular carriers. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-

20 dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive

25 water-insoluble matrices such as cyanogen bromide-activated carbo

hydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

30   Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecule Properties, W.H. Freeman &

35 Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

- 30 -

Such derivatized moieties may improve the
solubility, absorption, biological half life, and the like.
The moieties may alternatively eliminate or attenuate any
undesirable side effect of the protein and the like. Moieties
5 capable of mediating such effects are disclosed, for example,
in Remington's Pharmaceutical Sciences, 16th ed., Mack
Publishing Co., Easton, PA (1980)

The preferred bacterial host for this invention is
E. coli. In other embodiments, other bacterial species can be
10 used. In yet other embodiments, eukaryotic cells may be
utilized, such as, for example, yeast, filamentous fungi, or
the like. Use of these cell types are well known in the art.
Any host may be used to express the protein which is
compatible with replicon and control sequences in the
15 expression plasmid. In general, vectors containing replicon
and control sequences are derived from species compatible with
a host cell are used in connection with the host. The vector
ordinarily carries a replicon site, as well as specific genes
which are capable of providing phenotypic selection in
20 infected or in transformed cells. The expression of the
fusion protein can also be placed under control with other
regulatory sequences which may be homologous to the organism
in its untransformed state.

This invention is also directed to an antibody
25 specific for an epitope of the GRB-1 or the GRB-2 protein and
the use of such an antibody to detect the presence of, or
measure the quantity or concentration of, the GRB protein in a
cell, a cell or tissue extract, or a biological fluid.

The term "antibody" is meant to include polyclonal
30 antibodies, monoclonal antibodies (mAbs), chimeric antibodies,
and anti-idiotypic (anti-Id) antibodies.

Polyclonal antibodies are heterogeneous populations
of antibody molecules derived from the sera of animals
immunized with an antigen.

35 Monoclonal antibodies are a substantially
homogeneous population of antibodies to specific antigens.
MAbs may be obtained by methods known to those skilled in the
art. See, for example Kohler and Milstein, Nature 256:495-497

(1975) and U.S. Patent No. 4,376,110. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof. The hybridoma producing the mAbs of this invention may be cultivated _in vitro_ or _in vivo_.

5 Production of high titers of mAbs _in vivo_ production makes this the presently preferred method of production. Briefly, cells from the individual hybridomas are injected intraperitoneally into pristane-primed BALB/c mice to produce ascites fluid containing high concentrations of the desired

10 mAbs. MAbs of isotype IgM or IgG may be purified from such ascites fluids, or from culture supernatants, using column chromatography methods well known to those of skill in the art.

Chimeric antibodies are molecules different portions

15 of which are derived from different animal species , such as those having variable region derived from a murine mAb and a human immunoglobulin constant region. Chimeric antibodies and methods for their production are known in the art (Cabilly _et al_, _Proc. Natl. Acad. Sci. USA_ _81_:3273-3277 (1984); Morrison

20 _et al._, _Proc. Natl. Acad. Sci. USA_ 81:6851-6855 (1984); Boulianne _et al._, _Nature_ _312_:643-646 (1984); Cabilly _et al._, European Patent Application 125023 (published November 14, 1984); Neuberger _et al._, _Nature_ _314_:268-270 (1985); Taniguchi _et al._, European Patent Application 171496 (published February

25 19, 1985); Morrison _et al._, European Patent Application 173494 (published March 5, 1986); Neuberger _et al._, PCT Application WO 86/01533, (published March 13, 1986); Kudo _et al._, European Patent Application 184187 (published June 11, 1986); Morrison _et al._, European Patent Application 173494 (published

30 March 5, 1986); Sahagan _et al._, _J. Immunol._ _137_:1066-1074 (1986); Robinson _et al._, International Patent Publication #PCT/US86/02269 (published 7 May 1987); Liu _et al._, _Proc. Natl. Acad. Sci. USA_ _84_:3439-3443 (1987); Sun _et al._, _Proc. Natl. Acad. Sci. USA_ _84_:214-218 (1987); Better _et al._, _Science_

35 _240_:1041- 1043 (1988)). These references are hereby incorporated by reference.

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with

- 32 -

the antigen-binding site of an antibody. An Id antibody can
be prepared by immunizing an animal of the same species and
genetic type (e.g. mouse strain) as the source of the mAb with
the mAb to which an anti-Id is being prepared. The immunized

5 animal will recognize and respond to the idiotypic
determinants of the immunizing antibody by producing an
antibody to these idiotypic determinants (the anti-Id
antibody).

The anti-Id antibody may also be used as an

10 "immunogen" to induce an immune response in yet another
animal, producing a so-called anti-anti-Id antibody. The
anti-anti-Id may be epitopically identical to the original mAb
which induced the anti-Id. Thus, by using antibodies to the
idiotypic determinants of a mAb, it is possible to identify

15 other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against the GRB protein
of the present invention may be used to induce anti-Id
antibodies in suitable animals, such as BALB/c mice. Spleen
cells from such immunized mice are used to produce anti-Id

20 hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs
can be coupled to a carrier such as keyhole limpet hemocyanin
(KLH) and used to immunize additional BALB/c mice. Sera from
these mice will contain anti-anti-Id antibodies that have the
binding properties of the original mAb specific for a GRB

25 protein epitope.

The anti-Id mAbs thus have their own idiotypic
epitopes, or "idiotopes" structurally similar to the epitope
being evaluated, such as GRB protein-α.

The term "antibody" is also meant to include both

30 intact molecules as well as fragments thereof, such as, for
example, Fab and $F(ab')_2$, which are capable of binding
antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of
intact antibody, clear more rapidly from the circulation, and
may have less non-specific tissue binding than an intact

35 antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)).

It will be appreciated that Fab and $F(ab')_2$ and
other fragments of the antibodies useful in the present
invention may be used for the detection and quantitation of

- 33 -

GRB protein according to the methods disclosed herein for
intact antibody molecules. . Such fragments are typically
produced by proteolytic cleavage, using enzymes such as papain
(to produce Fab fragments) or pepsin (to produce $F(ab')_2$
5 fragments).

An antibody is said to be "capable of binding" a
molecule if it is capable of specifically reacting with the
molecule to thereby bind the molecule to the antibody. The
term "epitope" is meant to refer to that portion of any
10 molecule capable of being bound by an antibody which can also
be recognized by that antibody. Epitopes or "antigenic
determinants" usually consist of chemically active surface
groupings of molecules such as amino acids or sugar side
chains and have specific three dimensional structural
15 characteristics as well as specific charge characteristics.

An "antigen" is a molecule or a portion of a
molecule capable of being bound by an antibody which is
additionally capable of inducing an animal to produce antibody
capable of binding to an epitope of that antigen. An antigen
20 may have one, or more than one epitope. The specific reaction
referred to above is meant to indicate that the antigen will
react, in a highly selective manner, with its corresponding
antibody and not with the multitude of other antibodies which
may be evoked by other antigens.

25   The antibodies, or fragments of antibodies, useful
in the present invention may be used to quantitatively or
qualitatively detect the presence of cells which express the
GRB protein. This can be accomplished by immunofluorescence
techniques employing a fluorescently labeled antibody (see
30 below) coupled with light microscopic, flow cytometric, or
fluorimetric detection.

The antibodies (of fragments thereof) useful in the
present invention may be employed histologically, as in
immunofluorescence or immunoelectron microscopy, for _in situ_
35 detection of GRB proteins. _In situ_ detection may be
accomplished by removing a histological specimen from a
patient, and providing the a labeled antibody of the present
invention to such a specimen. The antibody (or fragment) is

- 34 -

preferably provided by applying or by overlaying the labeled
antibody (or fragment) to a biological sample. Through the
use of such a procedure, it is possible to determine not only
the presence of the GRB protein but also its distribution on
5 the examined tissue. Using the present invention, those of
ordinary skill will readily perceive that any of a wide
variety of histological methods (such as staining procedures)
can be modified in order to achieve such _in situ_ detection.

    Such assays for GRB protein typically comprises
10 incubating a biological sample, such as a biological fluid, a
tissue extract, freshly harvested cells such as lymphocytes or
leucocytes, or cells which have been incubated in tissue
culture, in the presence of a detectably labeled antibody
capable of identifying GRB protein, and detecting the antibody
15 by any of a number of techniques well-known in the art.

    The biological sample may be treated with a solid
phase support such as nitrocellulose, or other solid support
which is capable of immobilizing cells, cell particles or
soluble proteins. The support may then be washed with
20 suitable buffers followed by treatment with the detectably
labeled GRB protein-specific antibody. The solid phase
support may then be washed with the buffer a second time to
remove unbound antibody. The amount of bound label on said
solid support may then be detected by conventional means.

25   By "solid phase support" is intended any support
capable of binding antigen or antibodies. Well-known
supports, or carriers, include glass, polystyrene,
polypropylene, polyethylene, dextran, nylon, amylases, natural
and modified celluloses, polyacrylamides, gabbros, and
30 magnetite. The nature of the carrier can be either soluble to
some extent or insoluble for the purposes of the present
invention. The support material may have virtually any
possible structural configuration so long as the coupled
molecule is capable of binding to an antigen or antibody.
35 Thus, the support configuration may be spherical, as in a
bead, or cylindrical, as in the inside surface of a test tube,
or the external surface of a rod. Alternatively, the surface
may be flat such as a sheet, test strip, etc. Preferred

supports include polystyrene beads. Those skilled in the art
will know many other suitable carriers for binding antibody or
antigen, or will be able to ascertain the same by use of
routine experimentation.

5   The binding activity of a given lot of anti-GRB-1
and anti-GRB-2 antibody may be determined according to well
known methods. Those skilled in the art will be able to
determine operative and optimal assay conditions for each
determination by employing routine experimentation.

10   Other such steps as washing, stirring, shaking, fil-
tering and the like may be added to the assays as is customary
or necessary for the particular situation.

           One of the ways in which the GRB-specific antibody
can be detectably labeled is by linking the same to an enzyme
15 and use in an enzyme immunoassay (EIA). This enzyme, in turn,
when later exposed to an appropriate substrate, will react
with the substrate in such a manner as to produce a chemical
moiety which can be detected, for example, by
spectrophotometric, fluorimetric or by visual means. Enzymes
20 which can be used to detectably label the antibody include,
but are not limited to, malate dehydrogenase, staphylococcal
nuclease, delta-5-steroid isomerase, yeast alcohol
dehydrogenase, alpha-glycerophosphate dehydrogenase, triose
phosphate isomerase, horseradish peroxidase, alkaline
25 phosphatase, asparaginase, glucose oxidase, beta-
galactosidase, ribonuclease, urease, catalase, glucose-6-
phosphate dehydrogenase, glucoamylase and
acetylcholinesterase. The detection can be accomplished by
colorimetric methods which employ a chromogenic substrate for
30 the enzyme. Detection may also be accomplished by visual
comparison of the extent of enzymatic reaction of a substrate
in comparison with similarly prepared standards.

           Detection may be accomplished using any of a variety
of other immunoassays. For example, by radioactively labeling
35 the antibodies or antibody fragments, it is possible to detect
R- PTPase through the use of a radioimmunoassay (RIA). A good
description of RIA may be found in Laboratory Techniques and
Bio chemistry in Molecular Biology, by Work, T.S., et al.,

- 36 -

North Holland Publishing Company, NY, (1978) with particular
reference to the chapter entitled "An Introduction to
Radioimmune Assay and Related Techniques" by Chard, T.,
incorporated by reference herein. The radioactive isotope can
5 be detected by such means as the use of a gamma counter or a
scintillation counter or by autoradiography.

It is also possible to label the antibody with a
fluorescent compound. When the fluorescently labeled antibody
is exposed to light of the proper wave length, its presence
10 can then be detected due to fluorescence. Among the most
commonly used fluorescent labelling compounds are fluorescein
isothiocyanate, rhodamine, phycoerythrin, phycocyanin,
allophycocyanin, o- phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using
15 fluorescence emitting metals such as ^{152}Eu, or others of the
lanthanide series. These metals can be attached to the
antibody using such metal chelating groups as
diethylenetriaminepentaacetic acid (DTPA) or
ethylenediaminetetraacetic acid (EDTA).

20   The antibody also can be detectably labeled by
coupling it to a chemiluminescent compound. The presence of
the chemiluminescent-tagged antibody is then determined by
detecting the presence of luminescence that arises during the
course of a chemical reaction. Examples of particularly
25 useful chemiluminescent labeling compounds are luminol,
isoluminol, theromatic acridinium ester, imidazole, acridinium
salt and oxalate ester.

Likewise, a bioluminescent compound may be used to
label the antibody of the present invention. Bioluminescence
30 is a type of chemiluminescence found in biological systems in
which a catalytic protein increases the efficiency of the
chemiluminescent reaction. The presence of a bioluminescent
protein is determined by detecting the presence of
luminescence. Important bioluminescent compounds for purposes
35 of labeling are luciferin, luciferase and aequorin.

The antibody molecules of the present invention may
be adapted for utilization in an immunometric assay, also
known as a "two-site" or "sandwich" assay. In a typical

immunometric assay, a quantity of unlabeled antibody (or
fragment of antibody) is bound to a solid support and a
quantity of detectably labeled soluble antibody is added to
permit detection and/or quantitation of the ternary complex
5   formed between solid-phase antibody, antigen, and labeled
antibody.

         Typical, and preferred, immunometric assays include
"forward" assays in which the antibody bound to the solid
phase is first contacted with the sample being tested to
10 extract the antigen from the sample by formation of a binary
solid phase antibody-antigen complex. After a suitable
incubation period, the solid support is washed to remove the
residue of the fluid sample, including unreacted antigen, if
any, and then contacted with the solution containing an
15 unknown quantity of labeled antibody (which functions as a
"reporter molecule"). After a second incubation period to
permit the labeled antibody to complex with the antigen bound
to the solid support through the unlabeled antibody, the solid
support is washed a second time to remove the unreacted
20 labeled antibody.

         In another type of "sandwich" assay, which may also
be useful with the antigens of the present invention, the so-
called "simultaneous" and "reverse" assays are used. A
simultaneous assay involves a single incubation step as the
25 antibody bound to the solid support and labeled antibody are
both added to the sample being tested at the same time. After
the incubation is completed, the solid support is washed to
remove the residue of fluid sample and uncomplexed labeled
antibody. The presence of labeled antibody associated with
30 the solid support is then determined as it would be in a
conventional "forward" sandwich assay.

         In the "reverse" assay, stepwise addition first of a
solution of labeled antibody to the fluid sample followed by
the addition of unlabeled antibody bound to a solid support
35 after a suitable incubation period is utilized. After a
second incubation, the solid phase is washed in conventional
fashion to free it of the residue of the sample being tested
and the solution of unreacted labeled antibody. The

- 38 -

determination of labeled antibody associated with a solid
support is then determined as in the "simultaneous" and
"forward" assays.

EXAMPLE I

5   A study was performed to determine the detectability
of binding of the C-terminal domain of EGFR to a protein
containing the SH2 domain immobilized on nitrocellulose
filters. For this purpose, the binding of the C-terminal
domain to a bacterially expressed fusion protein was assessed
10 (see Figure 1).

A.   Isolation and Labelling of the Carboxyterminal Domain of
the EGFR

         The intracellular portion of the EGFR, which
includes the tyrosine kinase domain and the carboxy terminal
15 domain, was purified from recombinant baculovirus which
expressed cDNA complementary to the intracellular domain of
the human EGFR, as described previously (Hsu, C-Y. et al.,
Cell Growth and Differentiation 1:191-200 (1990)). The
recombinant protein (2 μg) was then phosphorylated with
20 [gamma-^{32}P]ATP (200 μCi, 6000 Ci/Mmol)., at 4°C in HNTG (20 mM
HEPES, pH 7.5, 150mM NaCl, 0.1% Triton X-100, and 10%
glycerol) buffer which contained 5mM MnCl$_2$. In order to
remove unincorporated [y-32P] ATP, the phosphorylated kinase
was diluted to 1 ml with 20 mM HEPES, pH 7.5, containing 100
25 μg BSA and then concentrated in a Centricon-10 to a volume cf
50 μl. This procedure was repeated 3 times resulting in the
removal of >99% of the unincorporated ATP. To separate the C-
terminal domain from the kinase domain, the concentrated
protein was then digested with cyanogen bromide (CNBr) in 70%
30 formic acid for 14 hours at room temperature (see also Example
VI, below). Samples were then washed three times with water,
dried and resuspended in binding buffer to a concentration of
2 x 10^6 cpm/ml.

- 39 -

B. Binding of the C-terminal Domain of the EGFR to
Bacterially Expressed TrpE/GAP-SH2 Fusion Protein Immobilized
on Nitrocellulose

        TrpE and TrpE/GAP-SH2 were obtained from the
5 laboratory of Dr. Tony Pawson and prepared as previously
   described (Moran, M.F. et al., Proc. Natl. Acad. Sci. USA
   87:8622-8626 (1990)). Filter binding studies were performed
   according to published methods (Schneider, W.J. et al., Proc.
   Natl. Acad. Sci. 76:5577-5581 (1979); Daniel, T.O. et al., J.
10 Biol. Chem. 258:4606-4611 (1983)) with minor modifications.
   Various concentrations of either bacterially expressed TrpE
   fusion protein or bacterial protein alone were spotted onto
   nitrocellulose filters. After blocking the filters for 1 hour
   at 4°C in PBS containing 5% Carnation dry milk, $_{32}$P-labelled
15 C-terminal domain of the EGFR was added and incubation was
   continued overnight at 4°C. After 24 hours, the
   nitrocellulose filters were washed 3 times at room
   temperature with PBS containing 0.2% Triton X-100. The
   filters were dried and exposed to Kodak XAR-5 film at -80°C.

20 C. Results

        The above method permitted detection of specific
   binding of the EGFR C-terminal domain to less than 5 ng of a
   bacterially expressed GAP-SH2 fusion protein. The binding was
   specific, since it required tyrosine phosphorylation of the
25 probe and did not occur when irrelevant proteins were applied
   to nitrocellulose filters.

        The demonstration that the EGFR C-terminal domain
   could bind specifically to an SH2-containing protein
   immobilized on nitrocellulose filters encouraged the present
30 inventors to apply this approach to the screening of lambda
   gt11 expression libraries with the goal of identifying novel
   EGFR binding proteins.

                            EXAMPLE II

   Screening of Expression Libraries and Isolation of a cDNA
35 Clone Encoding a Novel SH2-Containing Protein

        The tyrosine phosphorylated C-terminal tail of the
   EGFR was used as a probe to screen expression libraries from

- 40 -

several different human tissues as described above. The
approach to screening is outlined in Figure 2. Numerous
positive clones have been identified so far using this
approach, of which two have been analyzed in detail.

5 A. Screening of cDNA Library

A lambda gt11, library, constructed from mRNA
isolated from human brain stem, was obtained from M. Jaye. To
screen the library, lambda gt11 phage were plated at a density
sufficient to produce 4 X 10^4 plaques per 150 mm agar plate.

10 A total of six plates were initially screened. After
incubation of the plates for 4 hours at 42°C, the plates were
overlaid with nitrocellulose filters which had been
impregnated with isopropyl-B-D-thiogalactopyranoside (IPTG),
as previously described (MacGregor, P.F. et al., Oncogene

15 5:451-458 (1990)). Incubation was continued overnight at
37°C. The filters were then removed, washed with tBST (10 mM
Tris-HCl, pH8, 150 mM NaCl, and 0.05% triton X-100) at room
temperature, and then blocked in HBB (20 mM HEPES, pH 7.5, 5
mM Mg/Cl, 1 mM KCl) buffer containing 5% carnation dry milk

20 for 1 hour at 4°C, as described (MacGregor et al., supra).
Following blocking, labelled tyrosine phosphorylated carboxy-
terminus (C-terminus) probe was added at a concentration of
1.6 X 10-4 µg/ml, and incubation was continued overnight. The
filters were then washed 3 times at room temperature in PBS

25 containing 0.2% Triton X-100. Filters were dried and exposed
to Kodak XAR-5 film at -80°C.

Agar plugs, corresponding to the positive clones,
were collect from the plates and placed in 1 ml of SM media.
After allowing the phages to diffuse from the agar, the phages

30 were replated and rescreened as described above. Those phages
that demonstrated enrichment on subsequent screening were
isolated and sequence. Lambda gt11 phage DNA was isolated by
the plate lysate method according to Maniatis et al., and
subcloned into EcoRI-digested M13 MP19 (Maniatis et al.,

35 1982). Single stranded DNA was isolated and sequenced by the
dideoxy chain termination method using the Sequenase DNA
sequencing kit (United States Biochemical).

- 41 -

In one experiment, 240,000 pfu from a human
brainstem lambda gt11 library were screened. A single plaque,
clone ki4 (Figure 3A) was isolated. On subsequent screening
this clone demonstrated enrichment, and on tertiary screening
5 all plaques bound the probe (Figure 3B). Clone ki4 contained
an insert of about 900 nucleotides, which, upon induction of
the lac promoter with IPTG, produced a fusion protein which
could bind the EGFR. The size of the fusion protein predicted
that the cDNA insert coded for a protein of about 300 amino
10 acids, which was the size expected if the cDNA contained a
single large open reading frame. To analyze clone ki4 in
more detail, DNA was isolated and the EcoRI fragment,
corresponding to the human cDNA insert, was subcloned into M13
and sequenced. Translation of the sequence from this insert
15 demonstrated a single large open reading frame
which, upon analysis using the Genbank database, was found to
contain a single stretch of about 100 amino acids with
sequence homology to SH2 domains of other known proteins
(Figures 4 and 5A). However, in other regions, no sequence
20 homology was noted. Thus, using this screening approach, a
new SH2-containing protein which could bind to the EGFR was
identified,.

B.   Isolated of Full Length cDNA
         The initial clone isolated encoded for an SH2
25 domain, but did not contain the 3' or 5' ends of the gene. To
isolated the full length cDNA, the library was rescreened
using DNA isolated from the initial positive phage. DNA, from
recombinant M13 bacteriophage which expressed the positive
clone, was amplified using a thermal cycler, Taq1 polymerase
30 and oligonucleotides complementary to the EcoR1 flanking
regions of the M13 vector. In order to obtain clones
containing more 5' sequence in information, a second amplified
DNA product, corresponding to the most 5' 250 nucleotides of
the initial isolated phage, was also generated by using
35 oligonucleotides complementary to sequences at both ends of
this region. [^{32}P]-labelled DNA probes were then prepared by
nick translation of the amplified products.

- 42 -

To rescreen the cDNA library, the library was
replated as described above. After incubation of the plates
for 8 hours at 37°C, the plates were cooled for 1 hour at 4°C
following which the phage DNA was transferred to
5 nitrocellulose filters. The filters were denatured in a
solution of 0.2 N NaOH and 1.5 M NaCl and then baked in vacuo
for 2 hours at 80°C (Sambrook, J. et al., (Molecular Cloning:
A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press,
Cold Spring Harbor, NY (1989)). After prehybridization of the
10 filters for 1 hour at 42°C, 32P-labelled DNA probe was added
and hybridization was continued overnight at 42°C in a
solution containing 5X Denhardt's, 50% formamide, 5X SSC, 0.1%
SDS, 200 mM Tris-HCl, pH 7.6 and 100 μg/ml salmon sperm DNA.
The filters were then washed in a solution containing 0.1X
15 SSC and 0.1% SDS, dried and exposed to Kodak XAR-5 film at -
70°C. Positive clones were then isolated and sequenced as
described above.

Since the insert from clone ki4 lacked the 3' and 5'
ends of the gene, the library was rescreened using two DNA
20 probes which were generated by amplifying DNA from clone ki4.
This approach enabled the identification of five additional
clones. Three of the clones extended 3' from the initial
clone ki4, two of which, clones, ki2.2 and ki2.4, contained a
polyadenylation signal and a long 3' untranslated region
25 (>1000 nucleotides). In addition, these clones encoded a
protein which contained a second SH2 domain (Figures 4 and
5A).

The other two clones, ki3.0 and ki5.3, extended 5'
from clone ki4. Both clones contained long open reading
30 frames and an AUG codon which met the translation initiation
criteria as defined by Kozak (Kozak, M. J. Cell. Biol.
108:229-241 (1989)). However, only clone ki3.0, when
translated into protein and compared with known sequences in
Genbank, was found to contain a domain of 50 amino acids which
35 was homologous to SH3 domains present in other known proteins.
The predicted molecular weight of the full length protein
encoded by the overlapping clones, ki2.2 and ki3.0, was about
84 kDa. This new protein was termed GRB-1.

- 43 -

EXAMPLE III
GRB-1 Protein Contains SH2 and SH3 domains

Analysis of the GRB-1 protein sequence by comparison
to sequences in the Genbank database revealed the presence of
two stretches of about 100 amino acids, starting at amino
acids 333 and 624, with sequence homology to SH2 domains of
other proteins known to interact with the EGFR (Figure 5A).
While GRB-1 displayed striking homology to other SH2 domains
at the protein level, it revealed no significant homology at
the DNA level. GRB-1 also contained a segment of about 50
amino acids, located in the N-terminal region, which had
sequence homology to SH3 domains (Figure 4 and 5B).

A comparison of the structural organization of GRB-1
with several other SH2/SH3 containing proteins is shown in
Figure 6. It is apparent from this scheme that the
localization of the SH2 and SH3 domains vary from protein to
protein. Despite this there are certain similarities and
differences among these SH2 containing proteins. GRB-1 is
similar to some other substrates which have been found to
interact with the EGFR, such as PLC-gamma and GAP, in that
GRB-1 contains two SH2 domains and a single SH3 domain.
However, unlike these substrates, GRB-1 contains no homology
to any known catalytic domain, and in this regard resembles
the protein encoded by the avian sarcoma virus, v-crk.

Out side of these regions there was no sequence
homology with other protein sequences present in Genbank. In
particular, GRB-1 lacked a consensus ATP-binding domain, and
did nod display sequence homology with any serine/threonine
kinase or tyrosine kinase.

The SH2 domain is thought to provide a common motif
by which enzymatically distinct signalling molecules can be
coupled to activated receptors with tyrosine kinase activity
(Moran, M.F. et al., Proc. Natl. Acad. Sci. USA 87:8622-8626
(1990); Anderson, D. et al., Science 250:979-982 (1990)).

The presence of SH2 domains in GRB-1 (Figure 4) and
in GRB-2 further reinforces the importance of this domain in

- 44 -

mediating the interaction of these proteins with the C-
terminal tail of the EGFR. Moreover, since many proteins
capable of interacting with tyrosine receptors remain to be
identified, this suggests that additional members of this
5 protein family remain to be discovered.

In addition to containing two SH2 domains, GRB-1
also contains an SH3 domain. The SH3 domain is a non-
catalytic domain of about 50 amino acid residues which is
shared among many SH2-containing proteins. Since SH3 domains
10 are also found in cytoskeletal proteins, such as spectrin and
fodrin, the function of this domain could be to localize these
proteins to the membrane or submembrane cytoskeleton where
they would interact with other molecules.

Comparison of the deduced amino acid sequence of
15 GRB-1 with the protein product encoded by the avian oncogene
v-crk may shed light on GRB-1 function. The gene v-crk
encodes a protein which is composed primarily of a viral gag
protein fused to an SH2 and SH3 domain (Mayer, B.J. et al.,
Nature 332:272-275 (1988)). Both GRB-1 and the p47$^{gag-crk}$
20 protein have no homology with any known catalytic domains.
However, chicken embryo fibroblasts transformed with p47^{gag-}
^{crk} display elevated levels of phosphotyrosine-containing
proteins (Mayer, B.J. et al., supra; Proc. Natl. Acad. Sci.
USA 87:2638-2642 (1990); Matsuda, M. et al., Science 248:1537-
25 1539 (1990)).

Since the v-crk product has been shown to bind
several phosphotyrosine-containing proteins in v-crk
transformed cells, it may be that the function of c-crk is to
act as a bridge between kinases and substrates. In this
30 regard, it is intriguing that GRB-1, like GAP and PLC-gamma,
contains two SH2 domains, the combination of which may be
ideally suited for linking other proteins to activated
tyrosine kinase receptors.

- 45 -

EXAMPLE IV
Northern Analysis of GRB-1 Expression

A. Methods

Total cellular RNA was prepared from monkey tissue
5 by the guanidinium isothiocyanate/cesium chloride method
described by Sambrook, J. et al., (supra). Poly (A)+ RNA was
prepared by oligo(dT) cellulose chromatography. For Northern
analysis, RNA was size fractionated by electrophoresis in a
1.2% agarose/2.2M formaldehyde gel, transferred onto a nylon
10 membrane by capillary action and baked at 80°C for 2 hours.
Following prehybridization, the blot was hybridized with a
$[^{32}P]$-nick-translated DNA probe which was prepared as descried
above. Hybridization was carried out overnight at 42°C in the
presence of 50% formamide, 5X SSC, 0.1% SDS, and 5X
15 Denhardt's. The membrane was then washed in 0.1X SSC, 0.1%
SDS at 42°C, and exposed to Kodak XAR film at -70°C for 12
hours using an intensifying screen.

B. Results

To test for the expression of mRNA corresponding to
20 the newly isolated cDNA, Northern blot analysis of different
monkey tissue mRNA, probed with DNA corresponding to the
insert from clone ki4, demonstrated the presence of two major
bands of 4.4 kb and 7.0 kb in most tissues examined (Figure
7). Expression was highest in the brain, with heart, spleen,
25 liver and thymus displaying decreasing levels of expression.
The 4.4 kb message corresponds to the expected size of the
transcript which would encode the isolated clones. In
contrast to the 4.4 and 7.0 kb transcripts observed in most
tissues, the skin contained two slightly smaller sized mRNAs
30 of 3.6 and 6.6 kb.

The 3.6, 6.6 and 7.0 kb transcripts may represent
alternatively spliced forms of mRNA, or may encode for
distinct but related mRNA species.

- 46 -

EXAMPLE V

Production of anti-GRB-1 Antibodies and Analysis of GRB-1
Fusion Protein

A. Methods

5   Polyclonal antibodies were produced by immunizing
rabbits with the ß-galactosidase fusion protein expressed by
the initial isolated phage clone, ki4. E. coli CAG 456
bacteria (obtained from Dr. Michael Snyder, Yale University)
were infected with recombinant phage ki4 at a multiplicity-of-
10 infection of 10 and ß-galactosidase fusion protein was
recovered from the protein pellet after 1.5 hours. Protein
extracts were prepared, separated on a 6% SDS-gel, and the
band corresponding to the fusion protein excised from gel and
used for immunization.

15   Human glioblastoma cell line U1242, rat bladder
carcinoma cell line NBT II, and NIH3T3 cells were grown to
confluence in DMEM medium supplemented with 10% fetal bovine
serum. Cells were labelled with [^{35}S]-methionine (50 μCi/ml)
in 0.5% fetal bovine serum and lysed after 12 hours as
20 previously described (Margolis, B. et al., Cell 57:1101-1107
(1989)). After immunoprecipitation with 10 μl of antibody
coupled to protein A-Sepharose, the beads were washed three
times with a solution containing 20mM HEPES, pH 7.5, 300mM
NaCl, 10% glycerol, 1% Triton X-100, 0.1% SDS, and 1% sodium
25 deoxycholate. After boiling in sample buffer proteins were
separated on a 8% SDS-gel.

B. Results

          Polyclonal antibodies were raised against the ß-
galactosidase fusion protein expressed by the initial isolated
30 phage. Immunoprecipitation experiments, using
biosynthetically labelled cells, demonstrated that these
antibodies recognized an 85 kDa protein in three different
cell lines (Figure 8, lanes designated "I"). Recognition of
the 85 kDa protein by this antiserum was specific since
35 preimmune serum did not recognize this protein (lanes
designated "P"). These results provided support for the
predicted molecular weight based on the amino acid sequence of
cloned GRB-1.

C.   Discussion

The finding that the gene for GRB-1 encodes for a
protein with an expected molecular weight of 85 kDa, together
with the demonstration that antibodies to GRB-1

5   immunoprecipitated an 85 kDa protein from three different cell
lines, suggest that GRB-1 may represent a particular protein
which had previously been shown to associate with activated
growth factor receptors, namely p85. While the exact function
of p85 was unknown, it was presumed to be phosphatidylinositol

10   (PI3)-kinase, since PI3-kinase activity copurified with an 85
kDa protein found in PDGF- stimulated as well as middle T-
antigen (MTAg)-transformed cells (Kaplan, D.R. _Cell_ _50_:1021-
1029 (1987); Whitman, M. _et al._, _Nature_ _315_:239-242 (1985);
Coughlin, S.R. _et al._, _Science_ _243_:1191-1194 (1989)). The

15   absence of an ATP binding site argues that GRB-1 is most
likely not a phospholipid kinase. GRB-1 exhibits 97% sequence
identity with murine and bovine p85. Hence, GRB-1 is the
human counterpart of p85. Recombinant p85 is able to bind to
the activated PDGFR or EGFR, but does not itself contain

20   intrinsic PI3 kinase activity. p85, however, is found
associated with a 110 kDa tyrosine phosphorylated protein
which may be the catalytic subunit of the PI3 Kinase. While
the exact relationship between PI3 kinase and p85 is not
known, overexpression of p85 modulates the interaction

25   between PI3 kinase and the PDGFR. p85 could function as a
regulatory subunit or as a bridge between activated receptors
and the PI3 kinase.

EXAMPLE VI

The Tyrosine Phosphorylated Carboxy-terminus of the
30   EGF Receptor is a Binding Site for GAP and PLC-gamma

The studies described below confirm that binding of
PLC-gamma and a fusion protein containing the SH2 and SH3
domains of GAP (trpE/GAP SH2) are specifically controlled by
autophosphorylation of the EGFR. The results show that

35   phosphorylation of PLC-gamma actually reduces its association
with the EGFR. Evidence is presented demonstrating that both
PLC-gamma and the trpE/GAP SH2 fusion protein bind

- 48 -

specifically to the tyrosine phosphorylated C-terminus of the
EGFR. In sum, these results indicate that the SH2/SH3 domains
interact directly with phosphotyrosine containing regions of
the EGF receptor.

5   A.   Materials and Methods
            1.   Cell lines, mutant receptors and fusion proteins
            The cell lines CD126 (Margolis, B.L. et al., J.
Biol. Chem. 264: 10667-10671 (1989a), HER14, K721 (Honegger,
A.M. et al., Cell 51: 199-209 (1987); Honegger, A.M. et al.,
10   Mol. Cell. Biol. 7:4567-4571 (1987)) were used as sources for
wild-type EGF receptor, kinase-negative (kin⁻) EGF receptor
and C-terminal (C-terminal) truncated EGF receptor,
respectively. The intracellular domain of the EGF receptor
(EGFR-C) was purified from a baculovirus expression system
15   (Hsu, C-.J. et al., Cell Growth Differ 1: 191-200 (1990))
(Figure 9A). 3TP1, a cell line which overexpresses
transfected PLC-gamma cDNA but has no EGF receptor was used as
a source of PLC-gamma (Margolis, B. et al., Science 248: 607-
610 (1990b)).
20   The preparation of trpE fusion proteins containing
the GAP SH2 domain (GAP residues 171-448, Figure 9B) has been
described by Moran, M.F. et al., Proc. Natl. Acad. Sci. USA
87: 8622-8626 (1990). Bacterial lysates containing trpE/GAP
SH2 fusion proteins were prepared by resuspending 1 g of
25   bacteria in 3 ml of 50 mM Tris pH 7.5, 0.5 mM EDTA, 0.1 mM
PMSF. After incubation at 4°C in 1 mg/ml lysozyme and 0.2%
NP-40, cells were sonicated 5 times for 5 seconds, and the
lysate was clarified by centrifugation for 30 min at 10,000 g.
Bacterial lysates were diluted 1:100 in the 1% Triton lysis
30   buffer with proteinase and phosphatase inhibitors as described
above and were precleared with protein A-Sepharose.
            2.   Antibodies, immunoprecipitation and immunoblotting
            The following anti-EGFR antibodies (Figure 9A) were
used: (a) mAb108, a monoclonal antibody directed against
35   domain III of the extracellular domain (Lax, I. et al., EMBO
J. 8: 421-427 (1989)); (b) antipeptide antibody RK2 specific
for residues 984-996; (c) antipeptide antibody C specific for
residues 1176-1186; and (d) antipeptide antibody F, specific

- 49 -

for residues 656-676. For immunoprecipitating the trpE fusion
proteins, a mouse monoclonal antibody against trpE (Oncogene
Science) bound to agarose linked anti-mouse IgG (Sigma) was
utilized. For immunoblotting, a polyclonal rabbit antibody
5   against trpE was used (Moran, M.F. et al., Proc. Natl. Acad.
Sci. USA 87: 8622-8626 (1990)). PLC-gamma was immunoblotted
and immunoprecipitated with a polyclonal rabbit anti-peptide
antibody described previously (Margolis, B. et al., Cell 57:
1101-1107 (1989b)).

10   The techniques used are described in several
references from the present inventors' laboratory (Margolis,
B.L. et al., J. Biol. Chem. 264: 10667-10671 (1989); Cell
57:1101-1107 (1989)). Unstimulated cells were grown to
confluence in Dulbecco's Modified Eagle Medium with 10% calf
15 serum and starved overnight in 1% fetal calf serum prior to
lysis in a 1% Triton X-100 lysis buffer containing proteinase
and phosphatase inhibitors. EGF receptors were
immunoprecipitated utilizing antibodies bound to protein A-
Sepharose. After washing the receptor material with HNTG (20
20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 10%
glycerol), autophosphorylation was induced by the addition of
5mM $MnCl_2$ and 30 μM ATP. Controls were incubated with Mn^{2+}
only. After further washes with HNTG, lysate containing
either PLC-gamma (from 3TP1 cells) or the bacterial fusion
25 proteins was added. After allowing binding to proceed for 90
min, three further washes with HNTG were performed and samples
were run on an SDS gel and immunoblotted.

3.   Cyanogen bromide (CNBr) cleavage

            EGFR-C was phosphorylated at 4°C with $MnCl_2$ and ATP
30 sometimes in the presence of [gamma-^{32}P]ATP (NEN/Dupont, 6000
Ci/mmol). The receptor preparation was then resuspended in 20
mM HEPES, pH 7.5, with 100 μg BSA and concentrated in a
Centricon 10 (Amicon) to 50 μl. Then 240 μl 88% formic acid
was added with two grains of CNBr and the samples were stored
35 under nitrogen in the dark for 14 h at room temperature.
Samples were dried and washed three times with water in a
Speed-Vac (Savant) and then resuspended in 1% Triton lysis
buffer.

- 50 -

B. <u>RESULTS</u>

A comparison was performed of the binding of PLC-gamma to wild-type and mutant EGFRs (Figure 9A). First, wild-type and mutant receptors from transfected NIH-3T3 cells were

5 immunoprecipitated and some of the receptor immunoprecipitates were allowed to undergo <u>in vitro</u> autophosphorylation with ATP and Mn^{2+} (Margolis, B. <u>et al.</u>, <u>Mol. Cell. Biol.</u> <u>10</u>: 435-441 (1990a)). Then, lysates from NIH-3T3 cells which overexpress PLC-gamma (Margolis, B. <u>et al.</u>, <u>Science</u> <u>248</u>: 607-610 (1990b))

10 were added and binding allowed to proceed for 90 min. at 4°C. After washing the immunoprecipitates with HNTG, the amount of PLC-gamma bound was assessed by immunoblotting.

As illustrated in Figure 10, PLC-gamma bound only to the tyrosine phosphorylated wild-type receptor but not to the

15 non-phosphorylated receptor.

To assess the importance of autophosphorylation, two studies with mutant receptors were then undertaken. First to be examined was the binding of PLC-gamma to a truncated EGF receptor missing 126 amino acids from the C-terminus (CD126,

20 Figure 9A) and devoid of four major autophosphorylation sites (Downward, J. <u>et al.</u>, <u>Nature</u> <u>311</u>: 483-485 (1984)). This truncated receptor was autophosphorylated, probably at tyrosine 992 (Walton, G.M. <u>et al.</u>, <u>J. Biol. Chem.</u> <u>265</u>: 1750-1754 (1990)). However, despite this level of tyrosine

25 autophosphorylation, the binding of PLC-gamma was markedly reduced compared to the full length receptor. Reduced association was also observed with CD63, a deletion mutant EGF receptor lacking 63 C-terminal residues containing two autophosphorylation sites. These results suggested a role for

30 the receptor C-terminus in either binding or modulating the binding of PLC-gamma to the EGF receptor.

Figure 10 also demonstrates that PLC-gamma cannot bind to the kin⁻ mutant receptor. To explore the importance of autophosphorylation in this effect, the kin⁻ receptor was

35 cross-phosphorylated with the CD126 receptor (Honegger, A.M. <u>et al.</u>, <u>Proc. Natl. Acad. Sci. USA</u> <u>86</u>:925-929 (1989)). This resulted in normalization of PLC-gamma binding to wild-type

- 51 -

levels. This suggested that phosphorylation of the kin⁻
receptor was sufficient to normalize binding to PLC-gamma.

To confirm that the kin⁻ receptor alone could bind
PLC-gamma after phosphorylation, this receptor was cross-
5 phosphorylated with a soluble, baculovirus-expressed EGFR
cytoplasmic domain (EGFR-C) that does not bind to the mAb 108
(Figure 9A).

Although cross-phosphorylation was not as strong as
with the CD126 mutant, tyrosine phosphorylation of the K721A
10 mutant and binding of PLC-gamma were clearly detected. This
finding confirms that tyrosine phosphorylation of the EGFR
promotes binding of PLC-gamma.

The role of PLC-gamma tyrosine phosphorylation in
the interaction between wild-type EGFR and PLC-gamma was
15 examined. Tyrosine phosphorylated PLC-gamma could be
dissociated from the EGFR more readily than non-phosphorylated
PLC-gamma (Figure 11), suggesting a lower affinity of tyrosine
phosphorylated PLC-gamma for the EGFR.

These findings were extended to examination of the
20 binding of a fusion protein containing trpE/GAP SH2 domain
(Figure 9B) to the baculovirus expressed EGFR-C. As with the
full length EGFR and PLC-gamma, the trpE/GAP SH2 fusion
protein domain bound only to the tyrosine phosphorylated EGFR-
C (Figure 12A). The trpE protein alone did not bind to EGFR-
25 C. Similarly, phosphorylated EGFR-C bound only to trpE/GAP
SH2; however, non-specific binding of non-phosphorylated EGFR-
C was high (Figure 12B). These results demonstrated that the
binding site of the EGFR is situated in its intracellular
domain.

30   In general, the trpE/GAP SH2 fusion protein bound
with a higher stoichiometry to full length EGFR than did PLC-
gamma. However, the fusion protein was not tyrosine
phosphorylated by the EGFR. The trpE/GAP SH2 protein much
better to the phosphorylated full length receptor compared to
35 the CD126 deletion mutant (Figure 13A). As shown in Figure
13B, cross-phosphorylation of the kin⁻ full length EGF
receptor by the EGFR-C allowed it to bind the trpE/GAP SH2
protein.

In control groups, the EGFR-C was shown not to enhance the binding to the CD126 receptor probably because this receptor was already maximally tyrosine phosphorylated (Figure 13A). Also, no binding was observed when EGFR-C was

5 tested in the presence of mAb 108 immunoprecipitate from cells containing no EGF receptor (Figure 13B). This indicates that the effects of EGFR-C could not be attributed to non-specific binding of tyrosine phosphorylated EGFR-C to sepharose. These studies confirm the importance of autophosphorylation in

10 mediating binding and show that for EGF receptor binding, the GAP SH2 domain behaves similarly to intact PLC-gamma.

The poor binding to the CD126 deletion mutant suggested that at least part of the binding site for the molecule was in the C-terminus. Yet an effect, possibly

15 allosteric, of this deletion on the overall conformation of the receptor could not be excluded. Therefore, the binding of PLC-gamma and trpE/GAP SH2 to a C-terminal fragment of the EGFR was examined. In the EGFR, the most C-terminal methionine residue is found at position 983; CNBr cleavage

20 therefore generates a 203 amino acid fragment which contains all the known autophosphorylation sites. This protein fragment is recognized by an antibody specific for the EGFR C-terminus, anti-C (Figure 9A).

When this C-terminal fragment was specifically

25 immunoprecipitated and tyrosine phosphorylated, it bound PLC-gamma and the trpE/GAP SH2 fusion protein (Figure 14). CNBr cleavage was complete; no full-length EGFR-C could be detected after proteolysis that could account for the binding. Again, no binding was seen to the non-phosphorylated C-terminal CNBr

30 fragment. CNBr cleavage of EGFR-C also generated a 97 amino acid N-terminal peptide identified by antibody F (Figure 9A, EGFR residues 645-742). This fragment, immunoprecipitated by antibody F, did not bind trpE/GAP SH2. Additionally, EGFR-C was autophosphorylated with [gamma-^{32}P]ATP and a ^{32}P-labeled

35 CNBr C-terminal fragment was generated. As shown in Figure 15, this fragment bound to the trpE/GAP SH2 fusion protein but not to trpE. In total, these findings demonstrate that direct binding to the tyrosine phosphorylated C-terminus contributes

- 53 -

at least in part to the specific binding of SH2 and SH3 domain proteins to the EGFR.

C.   Discussion

When taken together, the above findings and several
5 additional lines of evidence argue strongly that the phosphotyrosine residues are part of the actual binding site of the EGFR for SH2 domains. First, P47$^{gag-crk}$ was found to bind to nearly all phosphotyrosine-containing proteins in v-crk transformed cells (Matsuda, M. et al., Science 248: 1537-
10 1539 (1990)). Second, mutations of two autophosphorylation sites on the PDGF receptor greatly decreased the binding of GAP (Kazlauskas, A. et al., Science 247: 1578-1581 (1990)). Finally, the results presented above demonstrate specific binding to the C-terminus of the EGFR only when
15 phosphotyrosine is present.   Thus, it is concluded that the phosphotyrosine residues either comprise a part of the binding site or locally alter the conformation of this region, allowing binding. It is unlikely that phosphotyrosine alone constitutes the binding site. For example, phosphotyrosine
20 alone cannot interfere with the binding of P47$^{gag-crk}$ to phosphotyrosine-containing proteins (Matsuda et al., supra). Additionally, PLC-gamma does not bind to activated all molecules that contain phosphotyrosine residues, such as the CSF-1 receptor (Downing, J.R. et al., EMBO J. 8:3345-3350
25 (1989)). Similarly, the binding of PLC-gamma to PDGFR does not appear to be identical to GAP binding; different SH2 and SH3 domain-containing proteins may have different binding specificities (Kazlauskas et al., supra).

The references cited above are all incorporated by
30 reference herein, whether specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the
35 spirit and scope of the invention and without undue experimentation.

- 54 -

While this invention has been described in
connection with specific embodiments thereof, it will be
understood that it is capable of further modifications. This
application is intended to cover any variations, uses, or
5 adaptations of the inventions following, in general, the
principles of the invention and including such departures from
the present disclosure as come within known or customary
practice within the art to which the invention pertains and as
may be applied to the essential features hereinbefore set
10 forth as follows in the scope of the appended claims. It is to
be understood that the phraseology or terminology employed
herein is for the purpose of description and not of
limitation.

WHAT IS CLAIMED IS:

     1.   A method for detecting expression in a cell of a protein which is capable of binding to a tyrosine-phosphorylated polypeptide portion of a receptor tyrosine kinase molecule, said method comprising:

    (a) contacting said cell, an extract thereof, a lysate thereof, or a supernatant thereof with a solid phase carrier, causing the binding of said protein to said carrier;

    (b) incubating said carrier-bound protein with said tyrosine-phosphorylated polypeptide, allowing said polypeptide to bind to said carrier-bound protein;

    (c) removing materials not bound to said carrier; and

    (d) detecting the presence or measuring the amount of said tyrosine-phosphorylated polypeptide bound to said carrier,

thereby detecting the expression of said protein.

     2.   A method according to claim 1 wherein said receptor tyrosine kinase is selected from the group consisting of epidermal growth factor receptor, platelet-derived growth factor receptor, fibroblast growth factor receptor, colony stimulating factor-1 receptor and insulin receptor.

     3.   A method according to claim 1 wherein said protein is of human origin.

     4.   A method according to claim 1 wherein said phosphorylated polypeptide is detectably labeled.

     5.   A method according to claim 4 wherein said detectable label is a radiolabel.

     6.   A method according to claim 5 wherein said radio label is ^{32}P.

- 56 -

7. A method for mapping to a human chromosome a gene encoding a protein which is capable of binding to a tyrosine-phosphorylated polypeptide portion of a receptor tyrosine kinase molecule, said method comprising:

5   (a)   infecting bacterial cells with a human gene expression library;

          (b)   detecting a clone expressing said protein using a method according to claim 1;

          (c)   sequencing the DNA of said clone; and

10   (d)   mapping said sequence to a human chromosome.

8. A polypeptide probe useful in the detection of the expression of a protein capable of binding to a tyrosine-phosphorylated polypeptide portion of a receptor tyrosine kinase, said probe comprising an amino acid sequence derived 15 from the tyrosine-phosphorylated portion of said receptor molecule, or a functional derivative thereof, wherein said sequence has at least one phosphorylated tyrosine residue and lacks the tyrosine kinase portion of said receptor, and wherein said probe is detectably labeled.

20   9. A probe according to claim 8 having between one and five phosphorylated tyrosine residues.

10. A probe according to claim 8 between 25 and 250 amino acid residues.

11. A probe according to claim 8 and wherein the 25 detectable label is a radioactive phosphorous atom of said phosphorylated tyrosine residue.

- 57 -

    12.   A method for preparing a probe according to claim 8, comprising:

    (a)   providing said receptor, or a genetically engineered receptor-like derivative in substantially pure form, wherein said receptor or said receptor-like derivative has both a tyrosine kinase domain and a tyrosine-phosphorylated domain, said tyrosine-phosphorylated domain including at least one tyrosine residue capable of being phosphorylated by said tyrosine kinase;

    (b)   incubating said receptor or receptor-like derivative with [gamma-^{32}P]adenosine triphosphate under conditions permitting phosphorylation of said tyrosine residue, causing phosphorylation of said tyrosine residue;

thereby producing said probe.

    13.   A method according to claim 12, comprising, after step (b),

    (c)   additionally treating said phosphorylated receptor molecule with an agent capable of cleaving said molecule between the tyrosine kinase domain and the tyrosine-phosphorylated domain.

    14.   A method according to claim 13 wherein said agent is cyanogen bromide.

    15.   A method according to claim 12, wherein said receptor-like derivative is a polypeptide encoded by a DNA molecule comprising a DNA sequence encoding tyrosine kinase, linked to a DNA sequence encoding a selective enzymatic cleavage site, linked to a DNA sequence encoding said carboxyterminal domain, and wherein said agent is an enzyme capable of cleaving at said cleavage site.

- 58 -

16. A method for purifying from a complex mixture a
protein which is capable of binding to a tyrosine-
phosphorylated polypeptide portion of a receptor tyrosine
kinase molecule, said method comprising:
5   (a)   contacting said complex mixture with a solid
                      phase carrier to which a probe according to
                      claim 8 is bound, allowing said protein to bind
                      to said probe;
                (b)   removing materials not bound to said carrier;
10   and
                (c)   eluting said bound protein from said carrier,
thereby purifying said protein.

17. A protein, GRB-1, having the amino acid
sequence shown in Figure 4.

15   18. A protein, GRB-2, having the amino acid
sequence shown in Figure 16.

19. A DNA molecule encoding a protein according to
claim 17, or a functional derivative thereof, wherein, when
said DNA molecule naturally occurs, it is substantially free
20 of the nucleotide sequences with which it is natively
associated.

20. A DNA molecule encoding a protein according to
claim 18, or a functional derivative thereof, wherein, when
said DNA molecule naturally occurs, it is substantially free
25 of the nucleotide sequences with which it is natively
associated.

21. A DNA molecule consisting essentially of the
nucleotide sequence shown in Figure 4.

22. A DNA molecule encoding a protein according to
30 claim 18, or encoding a functional derivative thereof.
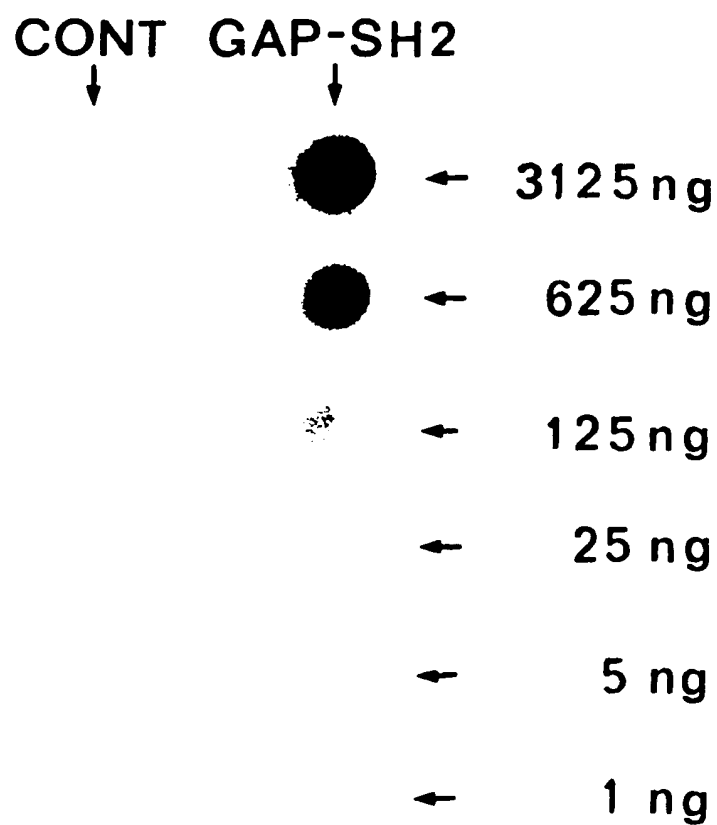
FIG. 1

CONT GAP-SH2

← 3125 ng

← 625 ng

← 125 ng

← 25 ng

← 5 ng

← 1 ng

FIG. 2

+[γ^{32}P] ATP

EGF - RECEPTOR
INTRACELLULAR DOMAIN

PLATE λgt11 LIBRARY

TRANSFER PLAQUES TO
NITROCELLULOSE

CNBr

HYBRIDIZE

32 P- LABELLED
CARBOXYTERMINUS

SELECT POSITIVE
COLONIES
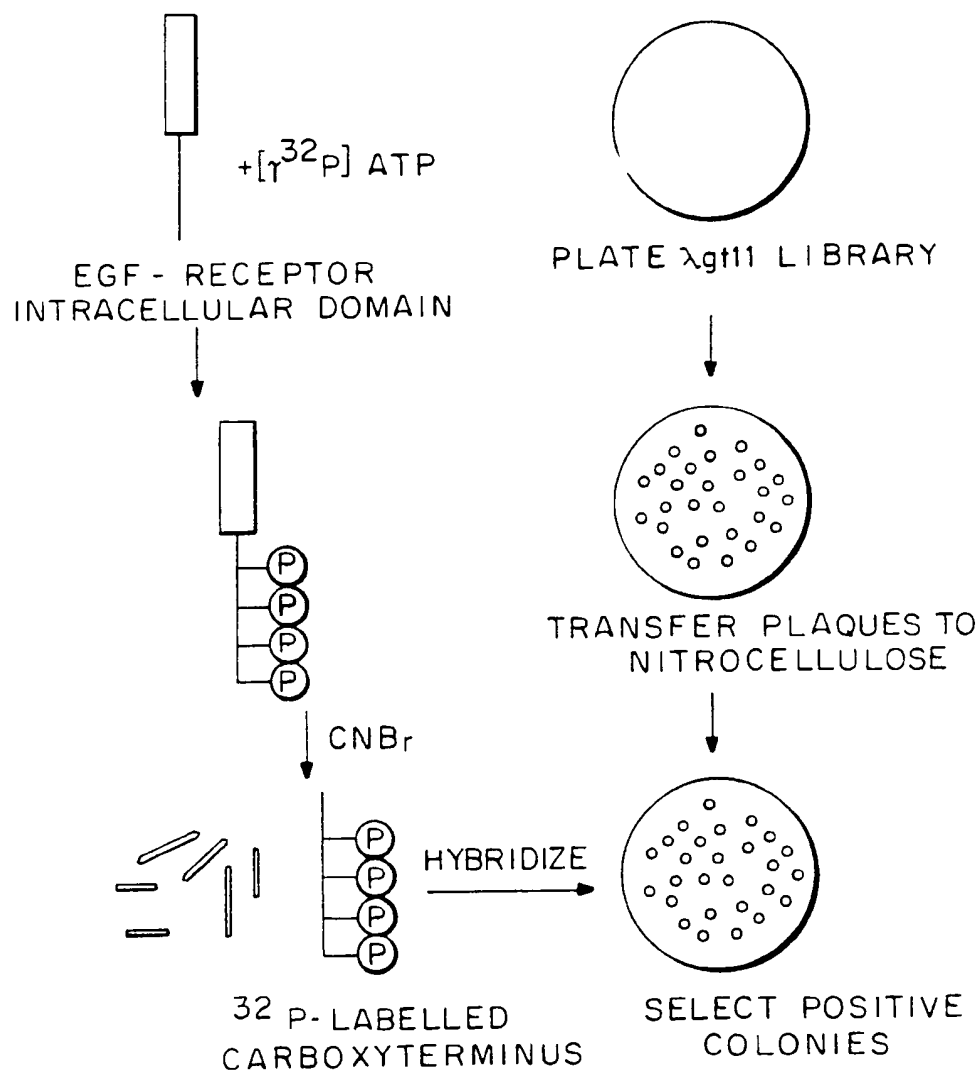
FIG. 3A
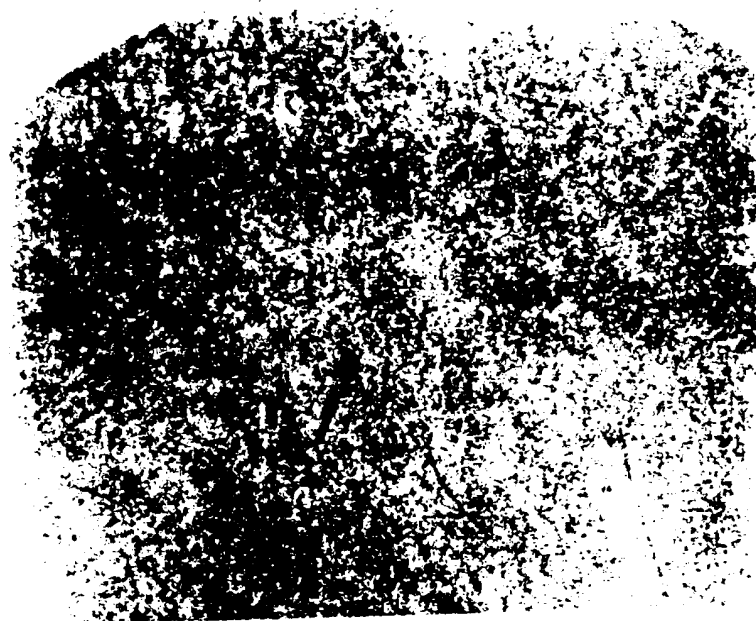
FIG. 3B

FIG. 4A

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                                                                    60
1   TACAACCAGGCTCAACTGTTGCATGGTAGCAGATTTGCAAACATGAGTGCTGAGGGGTAC
    ---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---+
    ATGTTGGTCCGAGTTGACAACGTACCATCGTCTAAACGTTTGTACTCACGACTCCCCATG
                                                   M  S  A  E  G  Y  -

                                                                    120
61  CAGTACAGAGCGCTGTATGATTATAAAAAGGAAAGAGAAGAGATATTGACTTGCACTTG
    ---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---+
    GTCATGTCTCGCGACATACTAATATTTTCCTTTCTCTTCTCTATAACTGAACGTGAAC
    Q  Y  R  A  L  Y  D  Y  K  K  E  R  E  E  D  I  D  L  H  L

                                                                    180
121 GGTGACATATTGACTGTGAATAAAGGGTCCTTAGTAGCTCTTGGATTCAGTGATGGACAG
    ---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---+
    CCACTGTATAACTGACACTTATTTCCCAGGAATCATCGAGAACCTAAGTCACTACCTGTC
    G  D  I  L  T  V  N  K  G  S  L  V  A  L  G  F  S  D  G  Q  -

                                                                    240
181 GAAGCCAGGCCTGAAGAAATTGGCTGGTTAAATGGCTATAATGAAACCACAGGGAAAGG
    ---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---+
    CTTCGGTCCGGACTTCTTTAACCGACCAATTTACCGATATTACTTTGGTGTCCCCTTTCC
    E  A  R  P  E  E  I  G  W  L  N  G  Y  N  E  T  T  G  E  R

                                                                    300
241 GGGGACTTTCCGGGAACTTACGTAGAATATATTGGAAGGAAAAAAATCTCGCCTCCCACA
    ---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---+
    CCCCTGAAAGGCCCTTGAATGCATCTTATATAACCTTCCTTTTTTTAGAGCGGAGGGTGT
    G  D  F  P  G  T  Y  V  E  Y  I  G  R  K  K  I  S  P  P  T

                                                                    360
301 CCAAAGCCCCGGCCACCTCGGCCTCTTCCGTTGCACCAGGTTCTTCGAAAACTGAAGCA
    ---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---+
    GGTTTCGGGGCCGGTGGAGCCGGAAGGACAACGTGGTCCAAGAAGCTTTTGACTTCGT
    P  K  P  R  P  P  R  P  L  P  V  A  P  G  S  S  K  T  E  A  -
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FIG. 4B

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361  GATGTTGAACAACAAGCTTTGACTCCTCCCGGATCTTGCAGAGAGCAGTTGCCCCTCCTGAC  420
     ----+----+----+----+----+----+
     CTACAACTTGTTGTTCGAAACTGAGAGGGCCTAGAACGTCTCGTCAAACGGGGAGGACTG
      D   V   E   Q   Q   A   L   T   L   P   D   L   A   E   Q   F   A   P   P   D   -

421  ATTGCCCCGCCTCTTCTTATCAAGCTCGTGGAAGCCATTGAAAGAAAGGTCTGGAATGT  480
     ----+----+----+----+----+----+
     TAACGGGGCGGAGAAGAATAGTTCGAGCACCTTCGGTAACTTTCTTTCCAGACCTTACA
      I   A   P   P   L   L   I   K   L   V   E   A   I   E   K   K   G   L   E   C   -

481  TCAACTCTATACAGAAACACAGAGCTCCAGCAACCTGGCAGAATTACGACAGCTTCTTGAT  540
     ----+----+----+----+----+----+
     AGTTGAGATATGTCTTTGTGTCTCGAGGTCGTTGGACCGTCTTAATGCTGTCGAAGAACTA
      S   T   L   Y   R   T   Q   S   S   S   N   L   A   E   L   R   Q   L   L   D   -

541  TGTGATACACCCTCCGTGGACTTGGAAATGATCGATGTGCACGTTTGGCTGACGCTTTC  600
     ----+----+----+----+----+----+
     ACACTATGTGGGAGGCACCTGAACCTTTACTAGCTACACGTGCAAAACCGACTGCGAAAG
      C   D   T   P   S   V   D   L   E   M   I   D   V   H   V   L   A   D   A   F   -

601  AAACGCTATCTCCTGGACTTACCAAATCCTGTCATTCCAGCAGCCGTTTACAGTGAAATG  660
     ----+----+----+----+----+----+
     TTTGCGATAGAGGACCTGAATGGTTTAGGACAGTAAGGTCGTCGGCAAATGTCACTTTAC
      K   R   Y   L   L   D   L   P   N   P   V   I   P   A   A   V   Y   S   E   M   -

661  ATTTCTTTAGCTCCAGAAGTACAAAGCTCCGAAGAATATATTCAGCTATTGAAGAAGCTT  720
     ----+----+----+----+----+----+
     TAAAGAAATCGAGGTCTTCATGTTTCGAGGCTTCTTATATAAGTCGATAACTTCTTCGAA
      I   S   L   A   P   E   V   Q   S   S   E   E   Y   I   Q   L   L   K   K   L   -
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FIG. 4C

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721 ATTAGGTCGCCTAGCATACCTCATCAGTATTGGCTTACGCTTCAGTATTTGTTAAAAACAT 780
    --------+---------+---------+---------+---------+---------+
    TAATCCAGCGGATCGTATGGAGTAGTCATAACCGAATGCGAAGTCATAAACAATTTGTA
  a  I  R  S  P  S  I  P  H  Q  Y  W  L  T  L  Q  Y  L  L  K  H

781 TTCTTCAAGCTCTCTCAAACCTCCAGCAAAAATCTGTTGAATGCAAGAGTACTCTCTGAA 840
    --------+---------+---------+---------+---------+---------+
    AAGAAGTTCGAGAGAGTTTGGAGGTCGTTTTTAGACAACTTACGTTCTCATGAGAGACTT
  a  F  F  K  L  S  Q  T  S  S  K  N  L  L  N  A  R  V  L  S  E

841 ATTTTCAGCCCTATGCTTTTCAGATTCTCAGCAGCCAGCTCTGATAATACTGAAAAACCTC 900
    --------+---------+---------+---------+---------+---------+
    TAAAAGTCGGGATACGAAAAGTCTAAGAGTCGTCGGTCGAGACTATTATGACTTTTGGAG
  a  I  F  S  P  M  L  F  R  F  S  A  A  S  D  N  T  E  N  L

901 ATAAAAGTTATAGAAATTTAATCTCAACTGAATGAATGAACGACAGCCTGCACCAGCA 960
    --------+---------+---------+---------+---------+---------+
    TATTTTGAATATGTTTAAAATTAGAGTTGACTTACCTTACTTGCTGTCGGACGTGGTCGT
  a  I  K  V  I  E  I  L  I  S  T  E  W  N  E  R  Q  P  A  P  A

961 CTGCCTCCTAAACCACCAAAACCTACTACTGTAGCCAACAACGGTATGAATAACAATATG 1020
    --------+---------+---------+---------+---------+---------+
    GACGGAGGATTTGGTGGTTTTGGATGATGACATCGGTTGTTGCCATACTTATTGTTATAC
  a  L  P  P  K  P  P  K  P  T  T  V  A  N  N  G  M  N  N  N  M

1021 TCCTTACAAAATGCTGAATGGTACTGGGGGAGATATCTCGAGGGAAGAAGTGAATGAAAAA 1080
     --------+---------+---------+---------+---------+---------+
     AGGAATGTTTTACGACTTACCATGACCCCTCTATAGAGCTCCCTTCTTCACTTACTTTTT
  a  S  L  Q  N  A  E  W  Y  W  G  D  I  S  R  E  E  V  N  E  K
```

FIG. 4D

```
1081 CTTCGAGATACAGCAGAGACGGGACCTTTTGGTACGAGATGCGTCACTAAAATGCATGGT
     ----+---------+---------+---------+---------+---------+ 1140
     GAAGCTCTATGTCGTCTCTGCCCTGGAAAAACCATGCTCTACGCAGATGATTTACGTACCA
a     L  R  D  T  A  D  G  T  F  L  V  R  D  A  S  T  K  M  H  G

1141 GATTATACTCTTACACTAAGGAAAGGGGGAAATAACAAATTAATCAAATATTCATCGA
     ----+---------+---------+---------+---------+---------+ 1200
     CTAATATGAGAATGTGATTCCTTTCCCCCTTTATTGTTTAATTAGTTTTATAAAGTAGCT
a     D  Y  T  L  T  L  R  R  K  G  G  N  N  K  L  I  K  I  F  H  R

1201 GATGGGAAATATGGCTTCTCTGACCCATTAACCTTCTGTGGTTGAATTAATAAAC
     ----+---------+---------+---------+---------+---------+ 1260
     CTACCCTTTATACCGAAGAGACTGGGTAATTGGAAGTCAAGACACCAACTTAATTATTTG
a     D  G  K  Y  G  F  S  D  P  L  T  F  S  S  V  V  E  L  I  N

1261 CACTACCGGAATGAATCTCTAGCTCAGTATAATCCCAAATTGGATGTGAAATTACTTTAT
     ----+---------+---------+---------+---------+---------+ 1320
     GTGATGGCCTTACTTAGAGATCGAGTCATATTAGGGTTTAACCTACACTTTAATGAAATA
a     H  Y  R  N  E  S  L  A  Q  Y  N  P  K  L  D  V  K  L  L  Y

1321 CCAGTATCCAAATACCAACAGGATCAAGTTGTCAAAGAAGATAATATTGAAGCTGTAGGG
     ----+---------+---------+---------+---------+---------+ 1380
     GGTCATAGGTTTATGGTTGTCCTAGTTCAACAGTTTCTTCTTATTATAACTTCGACATCCC
a     P  V  S  K  Y  Q  Q  D  Q  V  V  K  E  D  N  I  E  A  V  G

1381 AAAAAATTACATGAATATAACACTCAGTTTCAAGAAAAAAGTCGAGAATATGATAGATTA
     ----+---------+---------+---------+---------+---------+ 1440
     TTTTTTAATGTACTTATATTGTGAGTCAAAGTTCTTTTTTCAGCTCTTATACTATCTTAAT
a     K  K  L  H  E  Y  N  T  Q  F  Q  E  K  S  R  E  Y  D  R  I
```

FIG. 4E

```
1441  TATGAAGAATATACCCGCACATCCCAGGAAATCCAAATGAAAAGGACAGCTATTGAAGCA  1500
      ---------+---------+---------+---------+---------+---------+
      ATACTTCTTATATGGGCGTGTAGGGTCCTTTAGGTTTACTTTTCCTGTCGATAACTTCGT
       Y  E  E  Y  T  R  T  S  Q  E  I  Q  M  K  R  T  A  I  E  A  -

1501  TTTAATGAAACCATAAAAATATTTGAAGAACAGTGCCAAGAGCGGTACAGCAAA        1560
      ---------+---------+---------+---------+---------+---------+
      AAATTACTTTGGTATTTTTATAAACTTCTTGTCACGGGTCTGGGTTCTCGCCATGTCGTTT
       F  N  E  T  I  K  I  F  E  E  Q  C  Q  T  Q  E  R  Y  S  K  -

1561  GAATACATAGAAAAGTTTAAACGTGAAGGCAATGAGAAAGAAATACAAAGGATTATGCAT  1620
      ---------+---------+---------+---------+---------+---------+
      CTTATGTATCTTTTCAAATTTGCACTTCCGTTACTCTTTCTTTATGTTCCTAATACGTA
       E  Y  I  E  K  F  K  R  E  G  N  E  K  E  I  Q  R  I  M  H  -

1621  AATTATGATAAGTTGAAGTCTCGAATCAGTGAAATTATTGACAGTAGAAGAAGATTGGAA  1680
      ---------+---------+---------+---------+---------+---------+
      TTAATACTATTCAACTTCAGAGCTTAGTCACTTTAATAACTGTCATCTTCTTCTAACCTT
       N  Y  D  K  L  K  S  R  I  S  E  I  I  D  S  R  R  R  L  E  -

1681  GAAGACTTGAAGAAGCAGGCAGCTGAGTATCGAGTATCGAGAAATTGACAAACGTATGAACAGCATT  1740
      ---------+---------+---------+---------+---------+---------+
      CTTCTGAACTTCTTCGTCCGTCGACTCTCTTAACTGTTTGCATACTGTTGCGTAA
       E  D  L  K  K  Q  A  A  E  Y  R  E  I  D  K  R  M  N  S  I  -

1741  AAACCAGACCTTATCCAGCTGAGAAAGACGAGAGACCAATACTTGATGTGGTTGACTCAA  1800
      ---------+---------+---------+---------+---------+---------+
      ATTGGTCTGGAATAGGTCGACTCTTTCTGCTCTCTCGTGGTTATGAACTACACCAACTGAGTT
       K  P  D  L  I  Q  L  R  K  T  R  D  Q  Y  L  M  W  L  T  Q  -
```

FIG. 4F

```
1801   AAAGGTGTTCGGCAAAAGAAGTTGAACGAGTGGTTGGGCAATGAAAAACACTGAAGACCAA   1860
       ------+---------+---------+---------+---------+---------+
       TTTCCACAAGCCGTTTTCTTCAACTTGCTCACCAACCCGTTACTTTTTGTGACTTCTGGTT
a        K   G   V   R   Q   K   K   L   N   E   W   L   G   N   E   N   T   E   D   Q

1861   TATTCACTGGTGGAAGATGATGAAGATTTGCCCCATCATGATGAGAAGACATGGAATGTT   1920
       ------+---------+---------+---------+---------+---------+
       ATAAGTGACCACCTTCTACTACTTCTAAACGGGGTAGTACTACTCTTCTGTACCTTACAA
a        Y   S   L   V   E   D   D   E   D   L   P   H   H   D   E   K   T   W   N   V    -

1921   GGAAGCAGCAACCGAAACAAAGCTGAAAACCTGTTGCGAGGGAAGCCGAGATGGCACTTTT   1980
       ------+---------+---------+---------+---------+---------+
       CCTTCGTCGTTGGCTTTGTTTCGACTTTTGGACAACGCTCCCTTCGGCTCTACCGTGAAAA
a        G   S   S   N   R   K   A   E   N   L   L   R   G   K   R   D   G   T   F    -

1981   CTTGTCCGGGAGAGCAGTAAACAGGGCTGCTATGCCTGCTCTCGTAGTGGTGACGGCGAA   2040
       ------+---------+---------+---------+---------+---------+
       GAACAGGCCCTCTCGTCATTTGTCCCGACGATACGGACGAGACATCACCACCTGCCGCTT
a        L   V   R   E   S   S   K   Q   G   C   Y   A   C   S   V   V   V   D   G   E    -

2041   GTAAAGCATTGTGTCATAAACAAAACAGCAACTGGCTATGGCTTTGCCGAGCCCTATAAC   2100
       ------+---------+---------+---------+---------+---------+
       CATTTCGTAACACAGTATTTGTTTTGTCGTTGACCGATACCGAAACGGCTCGGGATATTG
a        V   K   H   C   V   I   N   K   T   A   T   G   Y   G   F   A   E   P   Y   N    -

2101   TTGTACAGCTCTCTGAAAGAACTGGTGCTACATTACCAACACACCTCCCTTGTGCAGCAC   2160
       ------+---------+---------+---------+---------+---------+
       AACATGTCGAGAGACTTTCTTGACCACGATGTAATGGTTGTGTGGAGGGAACACGTCGTG
a        L   Y   S   S   L   K   E   L   V   L   H   Y   Q   H   T   S   L   V   Q   H    -
```

FIG. 4G

```
        AACGACTCCCTCAATGTCACACTAGCCTACCCAGTATATGCACAGCAGAGGCGATGAAGC
2161    ---------+---------+---------+---------+---------+---------+    2220
        TTGCTGAGGGAGTTACAGTGTGATCGGATGGGTCATATACGTGTCGTCTCCGCTACTTCG

        N   D   S   L   N   V   T   L   A   Y   P   V   Y   A   Q   Q   R   R   -

        GCTTACTCTTTGATCCTTCCTGAAGTTCAGCCACCCTGAGGCCTCTGGAAAGCAAAGG
2221    ---------+---------+---------+---------+---------+---------+    2280
        CGAATGAGAAACTAGGAAGAGACTTCAAGTCGGTGGGACTCCGGAGACCTTTCGTTTCC

        GCTCCTCCTCCAGTCTGATCTGTGAATTGAGCTGCAGAAACGAAGCCATCTTTCTTTGAT
2281    ---------+---------+---------+---------+---------+---------+    2340
        CGAGGAGAGGTCAGACTAGACACTTAACTCGACGTCTTTGCTTCGGTAGAAAGAAACCTA

        GGGACTAGAGAGCTTTCTTTCACAAAAAGAGAAGTAGGGGAAGACATGCAGCCTAAGGCTGTA
2341    ---------+---------+---------+---------+---------+---------+    2400
        CCCTGATCTCGAAAGAAAGTGTTTTTCTTCATCCCCTTCGTACGTCGGATTCCGACAT

        TGATGACCACACGTTCCTAAGCTGGAGTGCTTATCCCTTCTTTTTCTTTTTTCTTTGGT
2401    ---------+---------+---------+---------+---------+---------+    2460
        ACTACTGGTGTGCAAGGATTCGACCTCACGAATAGGAAGAAAAGAAAAAAAGAAACCA

        TTAATTTAAAGCCACAACCACATACAACACAAAGAGAAAAAGAGAAAATGCAAAAATCTGC
2461    ---------+---------+---------+---------+---------+---------+    2520
        AATTAAATTTCGGTGTTGGTGTATGTTGTGTTTCTCTTTTCTTTACGTTTTTAGAGACG

        GTGCAGGGACAAAGAGGCCTTTAACCATGGTGCTTGTTAATGCTTTCTGAAGCTTTACCA
2521    ---------+---------+---------+---------+---------+---------+    2580
        CACGTCCCTGTTTCTCCGGAAATTGGTACCACGAACAATTACGAAAGACTTCGAAATGGT
```

FIG. 4H

```
2581   GCTGAAAGTTGGGACTCTCGGAGAGGCGGAGAGAGAGGCAGAGAAGAACCCTGGCCTGAGA
       ------+---------+---------+---------+---------+---------+ 2640
       CGACTTTCAACCCTGAGACCTCGCCGCTCCTCCTCCGTCTTCCGTCTTGGGACCGGACTCT

2641   AGGTTTGGTCCAGCCTGGTTTAGCCTGGATGTTGCTGTGCACGGTGGACCCAGACACATC
a      ------+---------+---------+---------+---------+---------+ 2700
       TCCAAACCAGGTCGGACCAAATCGGACCTACAACGACACGTGCCACCTGGGTCTGTGTAG

2701   GCACTGTGGATTATTCATTTGTAACAAATGAACGATATGTAGCAGAAAGGCACGTCCA
a      ------+---------+---------+---------+---------+---------+ 2760
       CGTGACACCTAATAAAGTAAACATTGTTTACTTGCTATACATCGTCTTTCCGTGCAGGT

2761   CTCACAAGGGACGCTTTGGGAGAATGTCAGTTCATGTGTATGTTCAGAAGAAATTCTGTCAT
a      ------+---------+---------+---------+---------+---------+ 2820
       GAGTGTTCCCTGCGAAACCCTCTTACAGTCAAGTACATACAAGTCTTCTTTAAGACAGTA

2821   AGAAAGTGCCAGAAAGTGTTTAACTTGTCAAAAAACAAAAACCCAGCAACAGAAAAATGG
a      ------+---------+---------+---------+---------+---------+ 2880
       TCTTTCACGGTCTTTCACAAATTGAACAGTTTTTTGTTTTGGGTCGTTGTCTTTTTACC

2881   AGTTTGGAAAACAGGACTTAAAATGACATTCAGTATATAAAATATGTACATAATATTGGA
a      ------+---------+---------+---------+---------+---------+ 2940
       TCAAACCTTTGTCCTGAATTTACTGTAAGTCATATATTTATACATGTATTATAACCT

2941   TGACTAACTATCAAATAGATGGATTTGTATCAATACCAAATAGCTTCTGTTTGTTTTGC
a      ------+---------+---------+---------+---------+---------+ 3000
       ACTGATTGATAGTTTATCTACCTAAACATAGTTTATGGTTTATCGAAGACAAAACAAAACG
```

F I G. 4 I

```
3001  TGAAGGCTAAATTCACAGGCGCTATGCAATTCTTAATTTCATTAAGTTGTTATTTCAGTT
      --------+---------+---------+---------+---------+---------+   3060
      ACTTCCGATTTAAGTGTCGCGATACGTTAAGAATTAAAAGTAATTCAACAATAAAGTCAA

3061  TTAAATGTACCTTCAGAATAAGCTTCCCACCCCAGTTTTTGTTGCTTGAAAATATTGTT
      --------+---------+---------+---------+---------+---------+   3120
      AATTTACATGGAAGTCTTATTCGAAGGGGTGGGGTCAAAAACAACGAACTTTATAACAA

3121  GTCCCGGATTTTGTTAATATTCATTTTTGTTATCCTTTTTAAAAATAAATGTACAGGA
      --------+---------+---------+---------+---------+---------+   3180
      CAGGGCCTAAAAAGAATTATAGTAAAAACAATAGGAAAAAATTTTATTTACATGTCCT

3181  TGCCAGTAAAAAAAAAAATGGCTTCAGAATTAAAACTATGAAATATTTTACAGTTTTCT
      --------+---------+---------+---------+---------+---------+   3240
      ACGGTCATTTTTTTTTACCGAAGTCTTAATTTGATACTTTATAAAATGTCAAAAAGA

3241  TGTACAGAGTACTTGCTGTTAGCCCAAGTTAAAAAGTTCATAACAGATTTTTTTGGAC
      --------+---------+---------+---------+---------+---------+   3300
      ACATGTCTCATGAACGACAATCGGGTTCCAATTTTCAAGTATTGTCTAAAAAAACCTG

3301  TGTTTTGTTGTGGGCAGTGCCTGATAAGCTTCAAAGCTGCTTATTCAATAAAAAAAAACC
      --------+---------+---------+---------+---------+---------+   3360
      ACAAAACAACCCGTCACGGACTATTCGAAGTTTCGACGAAATAAGTTATTTTTTTTTGG

3361  CGAATTCACTGG
      --------+--   3372
      GCTTAAGTGACC
```
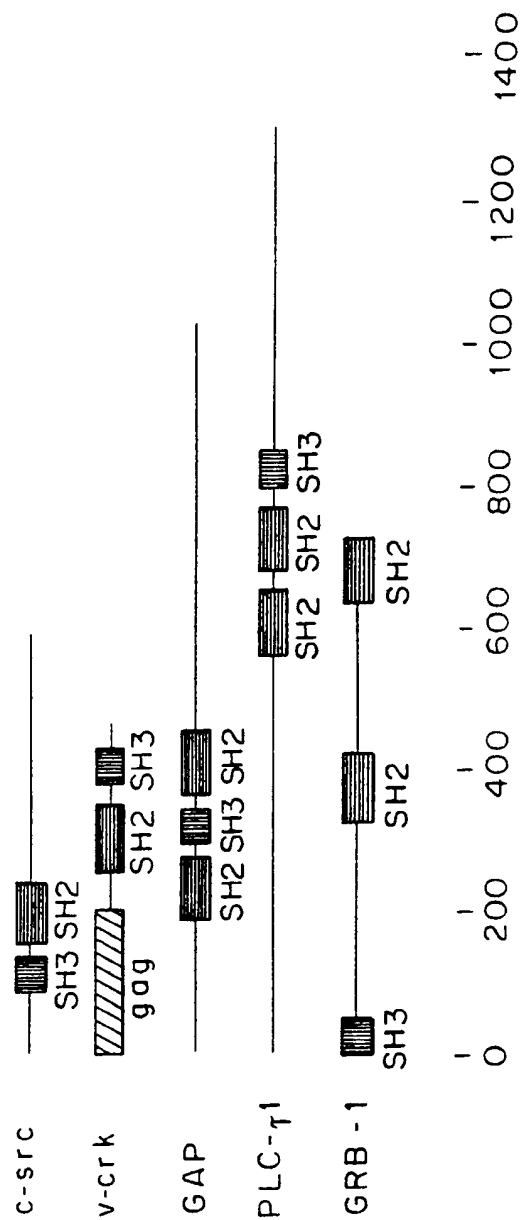
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FIG. 5

```
GRB-1 N 333  WYWGDIS -- R EE--- VN E- KL RDTAD-----    GTFLVRDST KM HGDY T LT LRK----- GG--NN LIKI
GRB-1 C 624  WNVGSSN -- R NK--- AE N- LL RGKRD-----    GTFLVRESS K- QGCY A CS VVV----- DG--EV KHCV

c-src 150    WYFGKIT -- R RE--- SE RL LL NPENPR-----    GTFLVRESE TT KGAY C LS VSDF-DNAKGLNVK HYKI
v-abl 248    WYHGPVS -- R NA--- AE YK KS SGIN-----      GSFLVRESE SS PG-Q R -S ISLRYE--G-RVY HYRI
PLC N 550    WFHGKLG AG R DGRHI AE R-- LL TEYCIETGAPD   GSFLVRESE TF VGDY T LS F--WR-N--G-KVQ HCRI
PLC C 668    WYHASLT -- R AQ--- AE H- ML MRVPRD-----    GAFLVRKRN -E PNSY A IS F--RAE--G-KIK HCRV
GAP N 178    WYHGKLD -- R TI--- AE E- RL RQAGKS-----    GSYLIRESD RR PGSF V LS FRSQM-N-V---VN HPRI
GAP C 348    WYHGKIS -- K QE--- AY N- LL MTVGQVC----   -SFLVRPSD NT PGDY S LY F-RTNENIQ--R-- -FKI
v-crk 248    WYWGRLS -- R GD--- AV S- LL QGQRH-----     GTFLVRDSG SI PGDF V LS VSES--S---RVS HYIV


GRB-1 N 384  --FHRD G KYGFSDPLT-----     F S SV V ELI N HY RNESLAQYNPKLDV- KL LY PVSK
GRB-1 C 672  INKTAT G -YGFAEPYNL-----    Y S SL K ELV L HY QHTSLVQHNDSLNV- TL AY PVYA

c-src 207    RKLDSG G FYITSRTQ-----      F S SL Q QLV A YY SKHADGLCH----- RL TN -VCP
v-abl 298    -NTASD G KLYVSSESR-----     F N TL A ELV H HH STVADGLITT----- LH YP --AP
PLC N 611    HSRQDA G TPKFFLTDNLV-----   F D SL Y DLI T HY QQVPLRCA-EFEM--- RL SL PV-P
PLC C 718    ---QQE G QTVMLGNSE-----     F D SL V DLI S YY EKHPLYRK---M-- KL RY PI--
GAP N 230    --IAMC G DYYIGGRR-----      F S SL S DLI G YY SHVSCLLKGE----- KL LY PVAP
GAP C 399    -CPTPN N QFMMGGRY-----      Y N SI G DII D HY RKEQIVEG-YY---- -L KE PV-P
v-crk 298    NSLGPA G GRRAGGEGPFAPGLNPTRFLIGDNV F D SL P SLL E FY KIHYLDT--TT----- -L IE PV--


GRB-1 10     ALYDY KKEREE D IDLHLGDI LT VNK G SLVALGFSDPEARPEDIG WL NGYNETTGER GDFP GT YVE YIGRK

c-src 88     ALYDY ESRTET D -------  LA FKK G ERLQIV----- -MNTEGD WW LAHSLTTGQT GYIP SN YVA PS-DS
v-abl 68     ALYDF VASGDN T -------  LS ITK G EKLRVLG----- -YNHNGE WC EAQTK-NGQ- GWVP SN YIT PV-NS
PLC 148      ALFDY KAGRED E -------  LT FTK S AIIQNV----- -EKQEGG WW RGDYHHKKQ- LWFP SN YVE EMV-S
GAP 284      AILDY TKVPDT D E------  IS FLK G DMFIVN----- -NELEDG WM WTNLRTDEQ GLIV ED LVE EV-GR
v-crk 375    ALFDF KGNDDG D -------  LP FKK G DILKIR----- -DKPEEQ WW NAEDMDGKR- GMIP VP YVE KCRPS
```
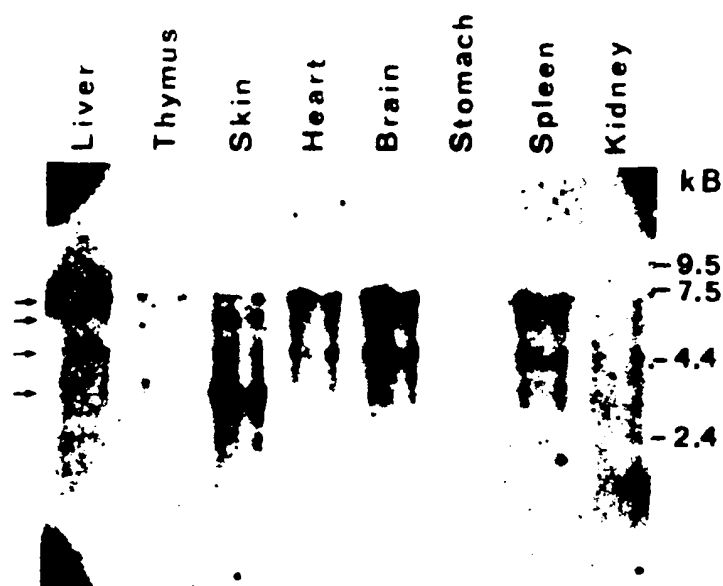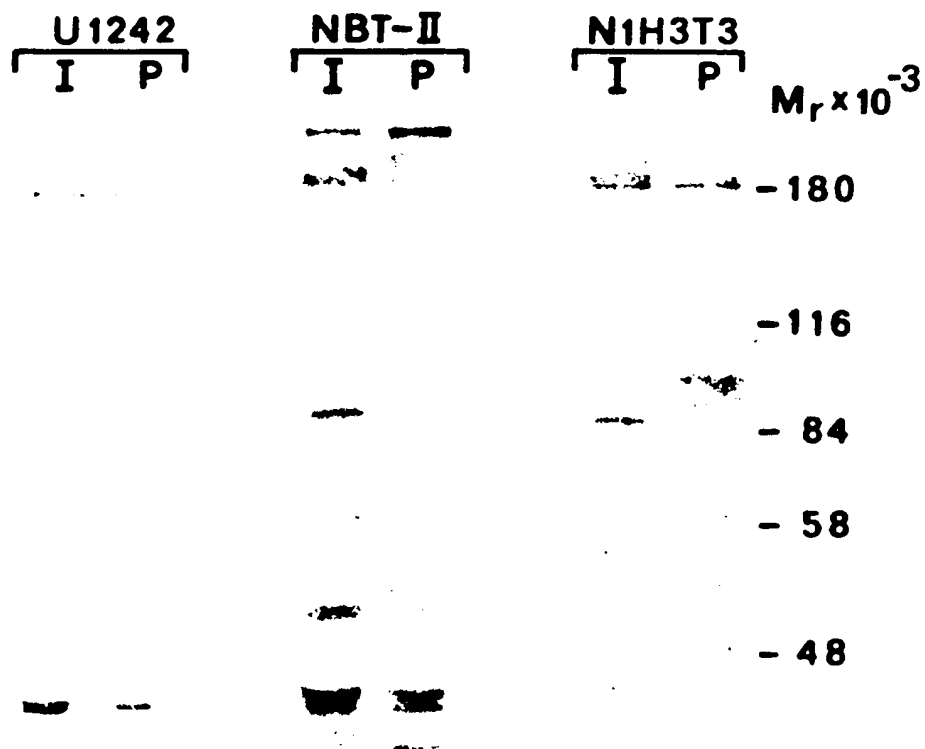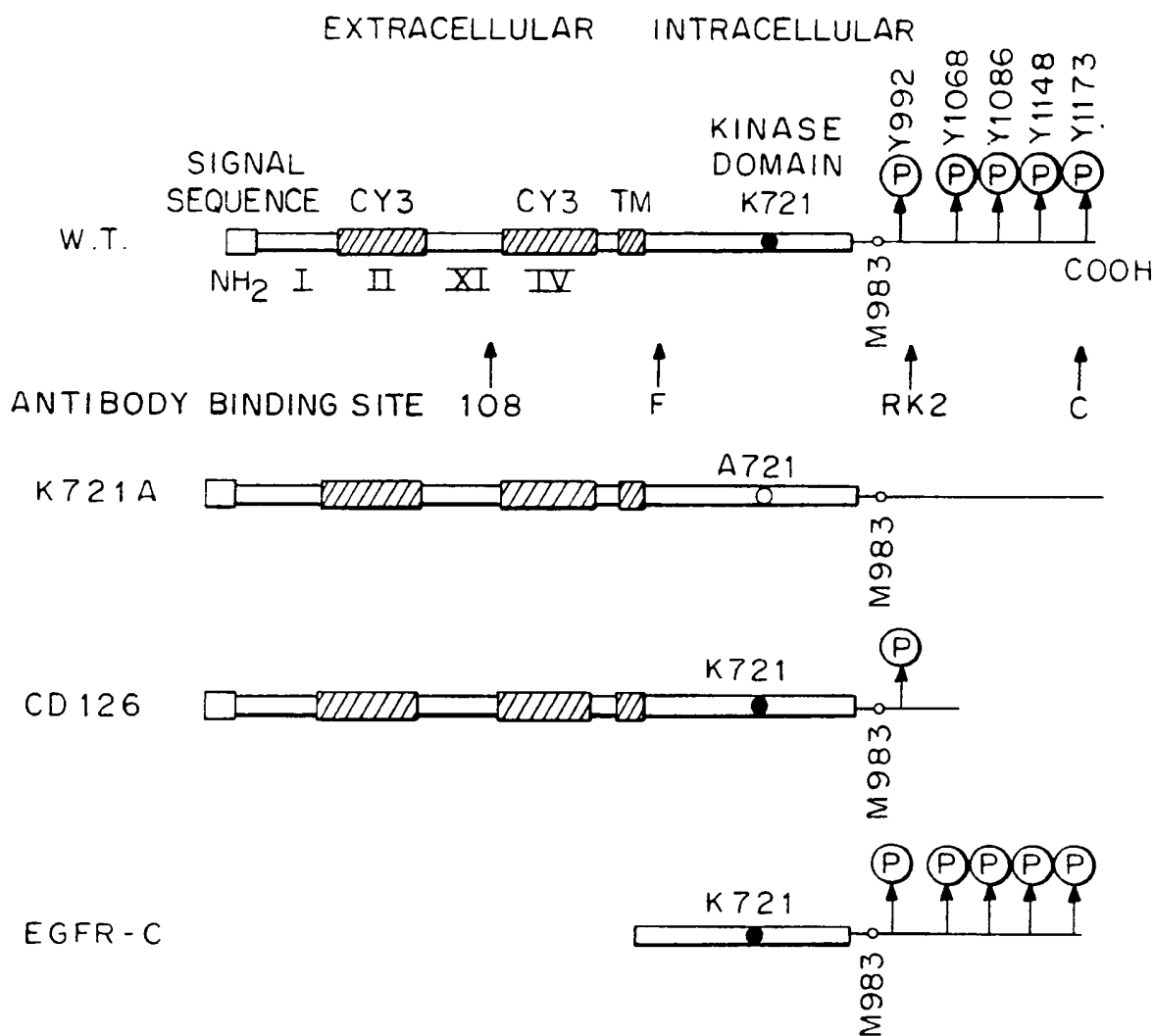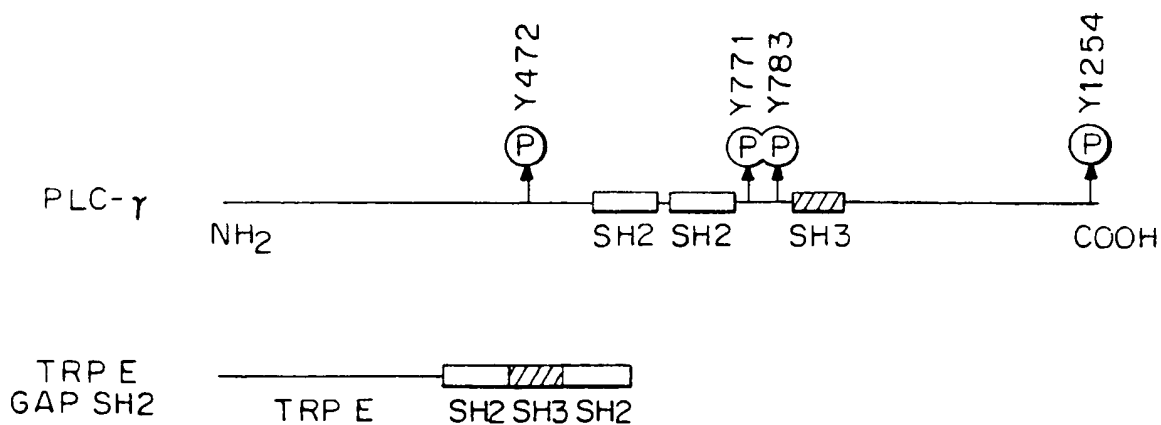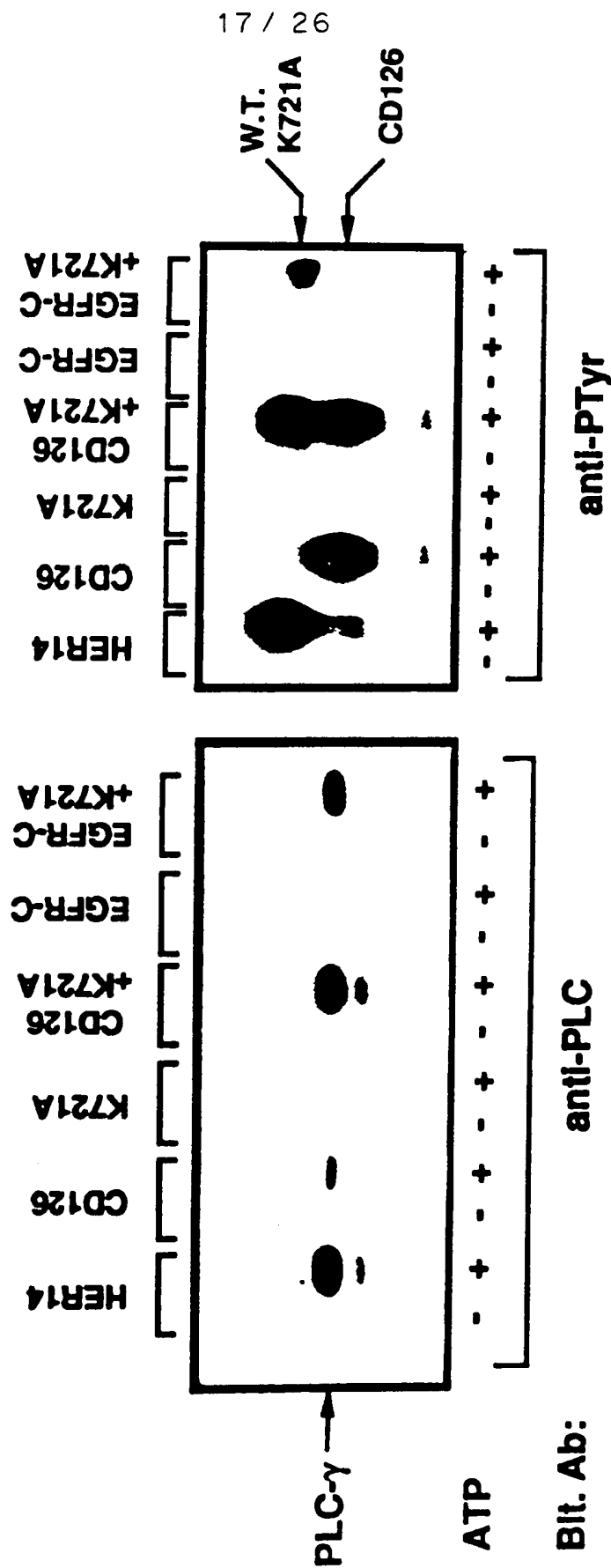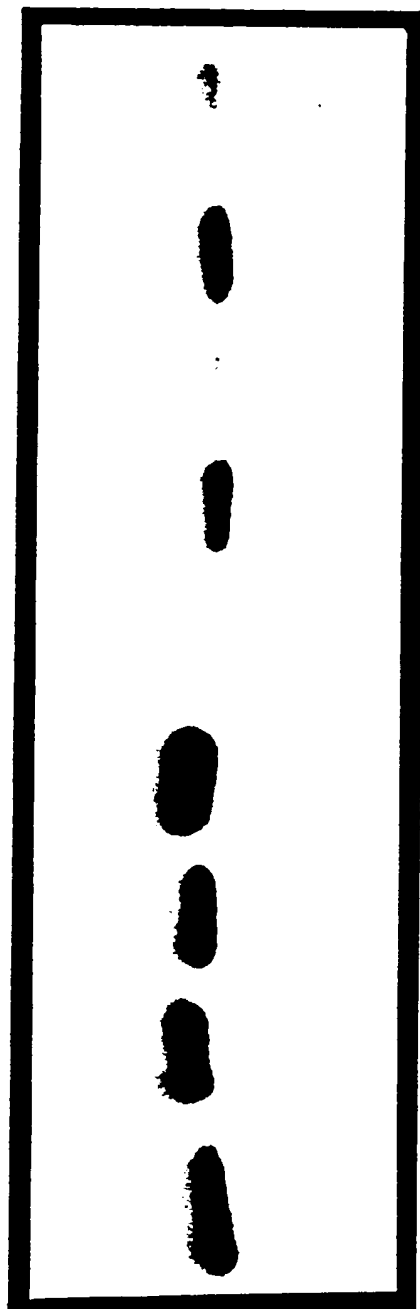
FIG. 6

F I G . 7

Liver   Thymus   Skin   Heart   Brain   Stomach   Spleen   Kidney   kB

— 9.5
— 7.5

— 4.4

— 2.4

F I G . 8

U1242		NBT-II		N1H3T3		
I	P	I	P	I	P	$M_r \times 10^{-3}$

—180

—116

— 84

— 58

— 48

FIG. 9A

EXTRACELLULAR   INTRACELLULAR

W.T.

SIGNAL
SEQUENCE   CY3   CY3   TM   KINASE DOMAIN
                                    K721

Y992 Y1068 Y1086 Y1148 Y1173

NH2   I   II   XI   IV

M983   COOH

ANTIBODY BINDING SITE   108   F   RK2   C

K721A   A721

M983

CD126   K721

M983

EGFR-C   K721

M983

FIG. 9B

Y472   Y771 Y783   Y1254

PLC-γ

NH2   SH2 SH2   SH3   COOH

TRP E
GAP SH2   TRP E   SH2 SH3 SH2

FIG. 10

FIG. 11

108

Imm.. Ab:

ATP

| | − | + | − | + | + | − | + |

PLC—Ⓟ
PLC

NO WASH   WASH

PLC

Blt. Ab:

FIG. 12 A

FIG. 12 B
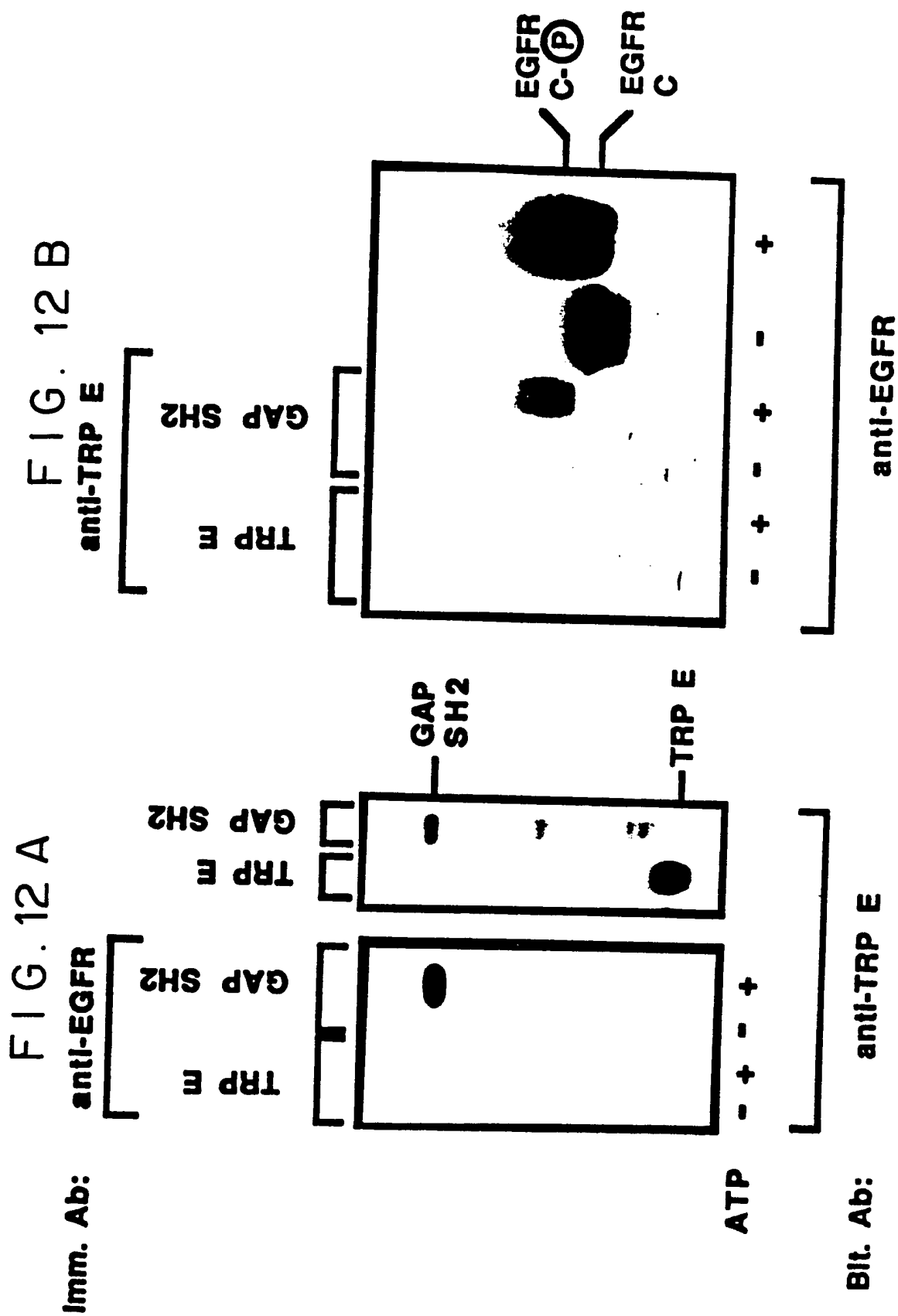
FIG. 13A

F I G . 13 B
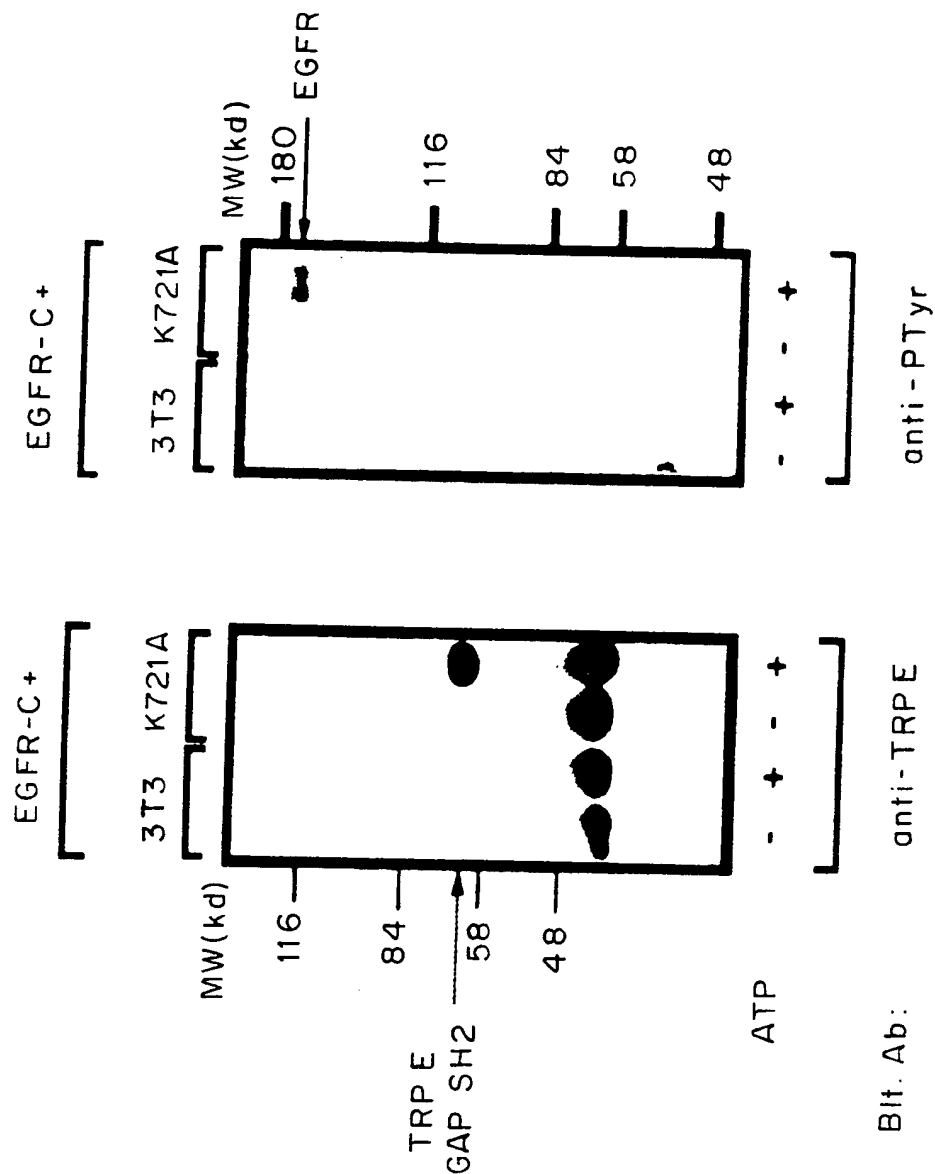
FIG. 14
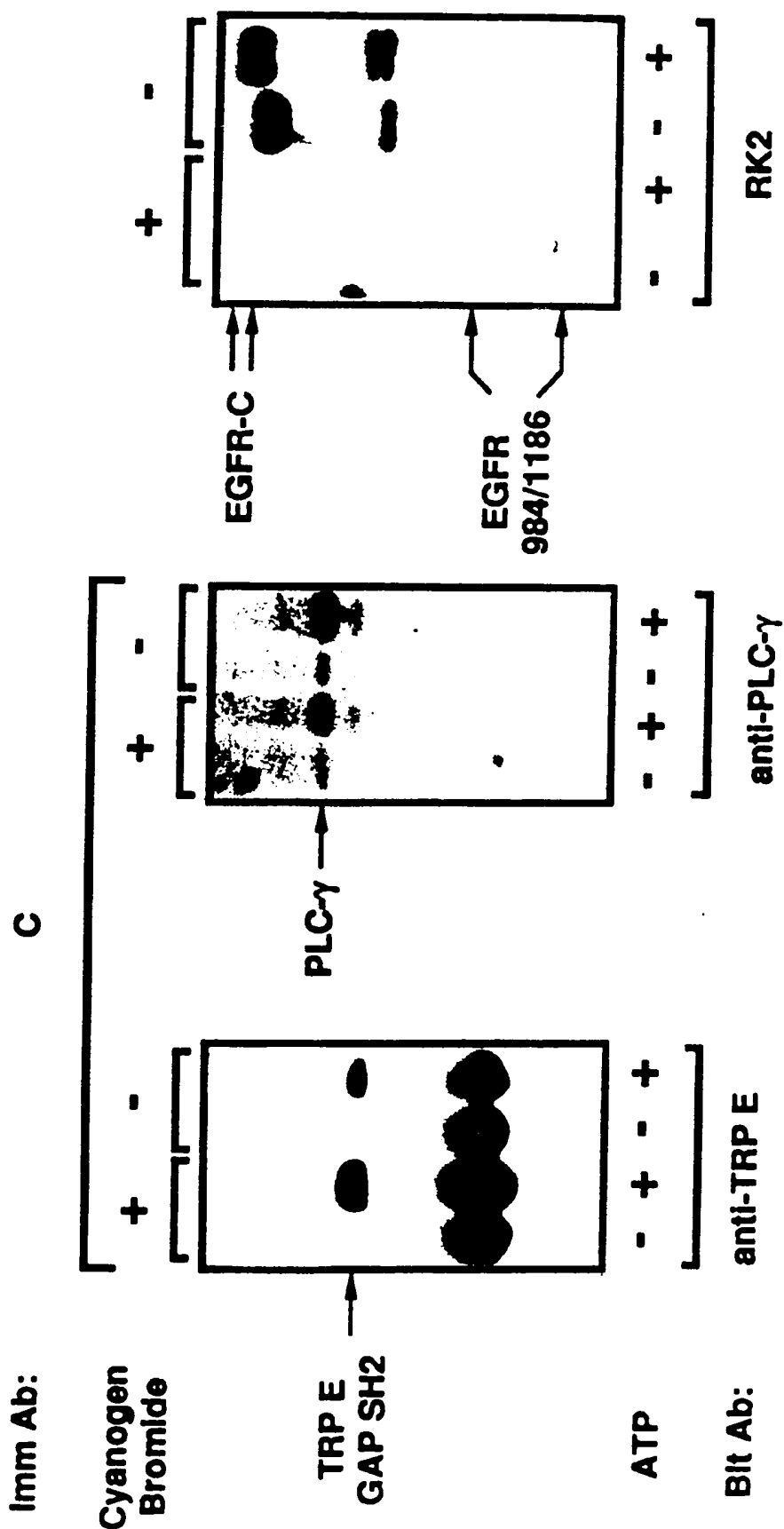
SUBSTITUTE SHEET

FIG. 15
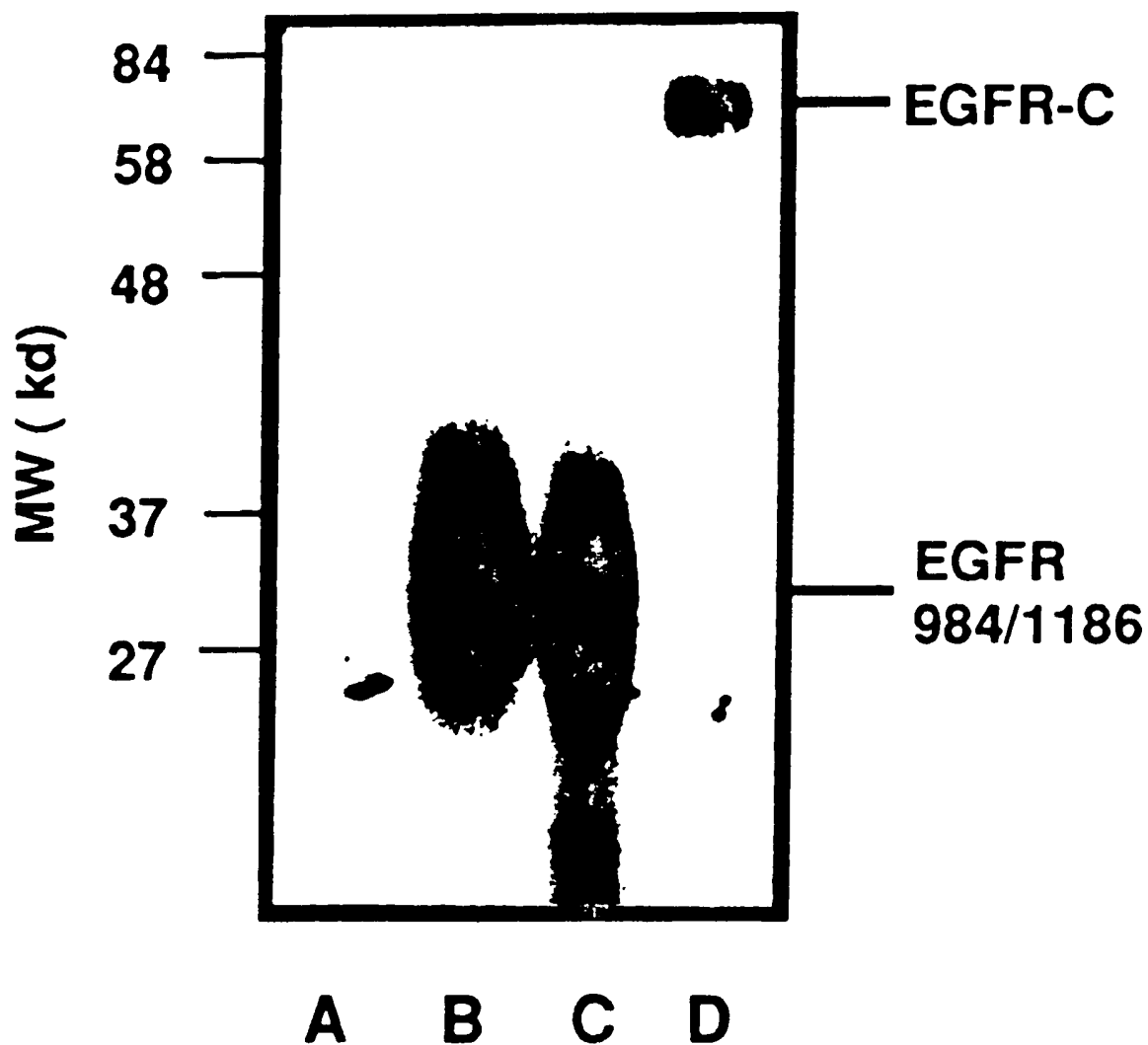
FIG. 16A

```
      GCCAGTGAATTCGGGCCCGAATTGGCAGAGCTTAATGAAAAGACGGCTTCATTCCCAAG   60
  1   ----+----+----+----+----+----+
      CGGTCACTTAAGCCCGGGCTTAACCGTCTCGAATTACCTTTCTGCCGAAGTAAGGGTTC
  a    A  S  E  F  G  P  E  L  A  E  L  N  G  K  D  G  F  I  P  K

      AACTACATAGAAATGAAACCACATCCGTGGTTTTTGGCAAAATCCCAGAGCCAAGGCA   120
 61   ----+----+----+----+----+----+
      TTGATGTATCTTTACTTTGGTGTAGGCACCAAAAAACCGTTTTAGGGTCTCGGTTCCGT
  a    N  Y  I  E  M  K  P  H  P  W  F  F  G  K  I  P  R  A  K  A
                            SH2 DOMAIN

      GAAGAAATGCTTAGCAAACAGCGGCACGATGGGGCCTTTCTTATCCGAGAGAGTGAGAGC  180
121   ----+----+----+----+----+----+
      CTTCTTTACGAATCGTTTGTCGCCGTGCTACCCCGGAAAGAATAGGCTCTCTCACTCTCG
  a    E  E  M  L  S  K  Q  R  H  D  G  A  F  L  I  R  E  S  E  S

      GCTCCCTGGGGACTTCTCCCCTCTCTGTCAAGTTTGGAACGATGTGCAGCACTTTCAAGGTG  240
181   ----+----+----+----+----+----+
      CCACCCCCTCAAACACGGAGAGACAGTTCAAACCTTGCTACACGTCGTGAAAGTTCCAC
  a    A  P  G  D  F  S  L  S  V  K  F  G  T  M  C  S  T  F  K  V

      CTCCCGAGATGGAGCCGGGAAGTACTTCCTCTGGTGGTGAAGTTCAATTCTTTGAATGAG  300
241   ----+----+----+----+----+----+
      GAGGGCTCTACCTCGGCCCTTCATGAAGGAGACCACCACTTAAGTTAAGAAACTTACTC
  a    L  P  R  W  S  R  E  V  L  P  L  V  V  K  F  N  S  L  N  E
```

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC (5): C07K 13/00, 15/28; C12N 15/62 US CL : 435/69.1, 252.3, 320.1; 530/350, 412

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/69.7, 252.3, 320.1; 530/350, 387

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]
APS, STN/MEDLINE search terms: TYROSINE KINASE#, ASSAY, EXPRESSION CLON?, LAMBDA GT11, ACTIVITY, ENZYMAT?.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category[*]	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
A	Proceedings of the National Academy of Sciences, Vol.87, issued November 1990, Moran et al,"Src homology region 2 domains direct protein-protein interactions in signal transduction", pages 8622 to 8626, see entire document.	1 to 28
A	METHODS IN ENZYMOLOGY, Vol.146, issued 1987, Pike,"Assay of Growth Factor-Stimulating Tyrosine Kinases Using Synthetic Peptide Substrates", pages 353 to 362, see entire document.	1 to 28

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
15 APRIL 1992	
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	JOHN D. ULM

Form PCT/ISA/210 (second sheet)(May 1986) ß

FIG. 16B

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301  CTGGTGGATTATCACAGATCTACACATCTGTCTCCAGAAACCAGCAGATATTCCTGCGGGAC  360
     ---------+---------+---------+---------+---------+---------+
     GACCACCTAATAGTGTCTAGATGTGTAGACAGAGTCTTTGGTCGTCTATAAGGACGCCCTG

a     L  V  D  Y  H  R  S  T  S  V  S  R  N  Q  Q  I  F  L  R  D  -

                                         $SH_2$ DOMAIN
361  ATAGAACAGGTGCCACAGCAGCCGACATACGTCCAGGCCCTCTTGACTTTGATCCCCAG  420
     ---------+---------+---------+---------+---------+---------+
     TATCTTGTCCACGGTGTCGTCGGCTGTATGCAGGTCCGGGAGAAACTAGGGGTC

a     I  E  Q  V  P  Q  Q  P  T  Y  V  Q  A  L  F  D  F  D  P  Q  -

421  GAGGATGGAGAGCTGGGCTTCCGCGGGGAGATTTTATCCATGTCATGATAACTCAGAC  480
     ---------+---------+---------+---------+---------+---------+
     CTCCTACCTCTCGACCCGAAGGCGCCCCTCTAAAATAGGTACAGTACCTATTGAGTCTG

a     E  D  G  E  L  G  F  R  R  G  D  F  I  H  V  M  D  N  S  D  -

481  CCCAACTGGTGGAAAGGAGCTTGCCACGGGCAGACCGGCATGTTTCCCGCGAATTATGT  540
     ---------+---------+---------+---------+---------+---------+
     GGGTTGACCACCTTTCCTCGAACGGTGCCCGTCTGGCCGTACAAAGGGGCGCTTAATACA

a     P  N  W  W  K  G  A  C  H  G  Q  T  G  M  F  P  R  E  L  C  -

541  CTCCCCCXGTGAACCGGAACGTCTAAGAGTCAAGAAGCAATTATTTAAAGAAAGTGAAAA  600
     ---------+---------+---------+---------+---------+---------+
     GAGGGGGXCACTTGGCCTTGCAGATTCTTCAGTTCTTCGTTAATAAATTTCTTTCACTTTT

a     L  P  ?  *  T  G  T  S  K  S  Q  E  A  I  I  *  R  K  *  K  -
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FIG.16C

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601  ATGTAAAACACATACAAAAGAATTAAACCACAAGCTGCCTCTGACAGCAGCCTGTGAGG
     ----|----|----|----|----|----|----|----|----|----|----|----+  660
     TACATTTGTGTATGTTTTCTTAATTTGGGTGTTCGACGGAGACTGCGTCGTCGGACACTCC
a       M  *  N  T  Y  K  R  I  K  P  T  S  C  L  *  Q  Q  P  V  R  -

661  GAGTGCAGAACACCTGGCGCGGGTCACCCTGTGACCCTCTCACTTTGGTTGGAACTTTAGG
     ----|----|----|----|----|----|----|----|----|----|----|----+  720
     CTCACGTCTTGTGGACCGCGCCCAGTGGGACACTGGGAGAGTGAAACCAACCTTGAAATCC
a       E  C  R  T  P  G  R  V  T  L  *  P  S  H  F  G  W  N  F  R  -

721  GGGTGGGAGGGGCGTTGGATTAAAAATGCCAAAACTTACCTATAAATTAAGAAGAGTT
     ----|----|----|----|----|----|----|----|----|----|----|----+  780
     CCCACCCTCCCCCGCAACCTAAATTTTACGGTTTGAATGGATATTTAATTCTTCTCAA
a       G  W  E  G  A  L  D  L  K  M  P  K  L  T  Y  K  L  R  R  V  -

781  TTTATTACAAATTTCACTGCTGCTCCTCCTTCCCCTCCTTGTCTTTTTTTTTCATCCT
     ----|----|----|----|----|----|----|----|----|----|----|----+  840
     AAATAATGTTTAAAAGTGACGACGAGGAGGAGAAAGGGAGGAAACAGAAAAAAAGTAGGA
a       F  I  T  N  F  H  C  C  S  S  F  P  S  F  V  F  F  F  H  P  -

841  TTTTTCTTCTTCGTCCATCAGTGCATGACGTTAAGGCCACGTATAGTCCTAGCTGACGC
     ----|----|----|----|----|----|----|----|----|----|----|----+  900
     AAAAAGAGAAGAAGACAGGTAGTCACGTACTGCAAATTCCGGTGCATATCAGGATCGACTGCG
a       F  F  S  S  V  H  Q  C  M  T  F  K  A  T  Y  S  P  S  *  R  -

901  CAATAATAAAAACCGAATTCGAGCTCGGGGATCCGGGGATCCTCTAGAGTC
     ----|----|----|----|----|----|----|----|----|----+  949
     GTTATTATTTTTGGCTTAAGCTCGAGCCCCTAGGCCCCTAGGAGATCTCAG
a       Q  *  *  K  P  N  S  S  S  D  P  G  I  L  *  S  ?  -
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